

**TISSUE-SPECIFIC AND PATHOGEN-SPECIFIC
TOXIC AGENTS AND RIBOZYMES**

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1. INTRODUCTION

The present invention relates to the discovery, identification, and characterization of toxic agents which are lethal to pathogens and methods for targeting and delivering such toxic agents to a pathogen or pathogen infected cell in order to treat and/or eradicate an infection. In particular, the present invention relates to toxic agents which target bacteria at different stages of the bacterial life cycle, which are delivered alone or in combination to bacteria or bacteria-infected cells. In particular, the invention relates to a phage delivery vehicle approach for the treatment of bacterial infections in humans and animals. The invention also relates to toxic agents which are lethal to diseased cells and methods for targeting such toxic agents to a diseased cell in order to treat and/or eradicate the disease. The present invention relates to promoter elements which are pathogen-specific. The invention also relates to promoter elements which are used to achieve pathogen-specific or tissue specific expression of the toxic agent(s) and/or ribozyme(s) of the present invention. Specifically, the invention relates to the delivery of one or more toxic gene products, antisense RNAs, or ribozymes, or combination thereof. The invention provides a novel system by which multiple pathogenic targets may be simultaneously targeted in order to kill a pathogen or pathogen-infected cell or render it less fit. Further, the invention has important implications in the eradication of drug-resistant bacterium and bacterial pathogens. The invention provides a novel system by which multiple targets may be simultaneously targeted to cause the death of a diseased cell or render it less fit. The invention has important implications in the eradication of drug-resistant pathogens (such as antibiotic resistant bacteria) and drug-resistant diseased cells (such as drug-resistant cancer cells).

2. BACKGROUND

2.1. ANTIMICROBIAL AGENTS

Infectious diseases sicken or kill millions of people each year. Each year in the United States alone, hundreds of thousands of people are infected with resistant bacterial strains that are no longer treatable with drugs like penicillin and vancomycin (Hiramatsu et al, 1997, Morbidity and Mortality Weekly Report 46:624-26). Infections associated with antimicrobial resistance include those acquired in hospitals (nosocomial), such as pneumonia particularly in the young, elderly and immunocompromised), typhoid fever, bacterial meningitis, and tuberculosis. Around the world, nearly 1.5 billion people carry

various types of the tuberculosis bacteria and depending on the country, up to 40 percent have proven to be resistant to antibiotics (see, Boyce et al, 1997, Epidemiology and prevention of nosocomial infections. In The Staphylococcus in Human Disease. Crossley and Archer Eds, Churchill Livingstone Inc., New York, NY). It is estimated that in some
5 developed countries, up to 60% of all nosocomial infections result from bacteria resistant to antibiotics. For example, *Pseudomonas aeruginosa*, is of the most common gram-negative bacterium associated with nosocomial infections and outbreaks in burn units. Infections by this organism are associated with high mortality (60%), which is attributed to the high intrinsic resistance of members of this genus to many structurally unrelated antibiotics.
10 Gram-positive bacteria also have a significant impact on infectious diseases. For example, *Staphylococcus aureus*, is a Gram-positive organism which is responsible for about 260,000 hospital acquired infections in the United States which subsequently causes between 60,000 and 80,000 deaths annually (see, Boyce et al, supra).

Although, numerous antimicrobial therapies have been designed to target
15 one or several infectious agents, many therapies show varying degrees of success in eradicating infection. Only a very limited number of new antibiotics have come onto the market in the last decade, yet the number of deadly bacteria that are resistant to these drug therapies has soared. For example, vancomycin is one of the last effective antimicrobial available for the treatment of methicillin-resistant *S. aureus* infection (MRSA). However,
20 vancomycin resistant isolates *S. aureus* have now emerged (Hiramatsu et al, 1997, Morbidity and Mortality Weekly Report 46:624-26). Additionally, the failure of many of these therapies to target specific infectious agents has lead to overuse or inappropriate use of the therapies, which in turn has lead to the development of drug resistant microbes. The development of drug resistance in many infectious agents has reduced the efficacy and
25 increased the risk of using the traditional antimicrobial therapies.

Accordingly, there is need in the art for novel molecules and novel combinations of molecules that can act as lethal agents in bacteria and which may be delivered to a pathogen, without causing toxicity to the infected host. Further, there is a need in the art for novel methods of targeting particular species of pathogens while leaving
30 the host's beneficial flora intact. The present invention provides such novel products, therapeutics, and methods for delivery which may be used as toxic agents against pathogens such as bacteria.

2.2. ANTISENSE

Antisense technology seeks to use RNA molecules which are complementary to (or antisense to) a cellular RNA, for the purpose of inhibiting a cellular RNA from being translated into the encoded protein. In this way, the expression of a specific protein is targeted for down regulation. However, a large number of difficulties exist in the art surrounding antisense technology. Commonly, delivery of an exogenous antisense molecule to the target cell is difficult or impossible to achieve. Further, antisense molecules do not consistently lead to a decrease in protein expression. For example, it has been shown that the expression of antisense RNA in transgenic mice did not invariably lead to a reduction in target RNA molecules, and when reduction in target RNA molecules did occur, it was not predictably paralleled by a reduction in protein. Even when protein levels were reduced sometimes no biological effect was detected (Whitton, J. Lindsay "Antisense Treatment of Viral Infection" *Adv. in Virus Res.* Vol. 44, 1994). Thus, there is a need in the art for a delivery system in which antisense molecules may be efficiently delivered to a target cell such as a bacterial pathogen.

2.3. RIBOZYMES

A ribozyme is a catalytic RNA molecule that cleaves RNA in a sequence specific manner. A key technical concern in the use of ribozymes as antimicrobial agents is that the ribozyme must be introduced into and expressed by the targeted microbe so that the ribozyme(s) can cleave the targeted RNA(s) inside the microorganism. A second important concern is the tight coupling of transcription and translation in microorganisms which can prevent binding to and cleavage of the bacterial RNA targets. Additionally, bacterial RNAs often have a shorter half life than eukaryotic RNAs, thus lessening the time in which to target a bacterial RNA. The invention described herein addresses these concerns and proves novel therapeutic treatments of bacterial infections using combinations of ribozymes and toxic agents.

3. SUMMARY OF THE INVENTION

The present invention provides toxic agents and methods for specifically targeting toxic agents to bacteria or bacteria-infected cells or other pathogens. Toxic agents of the present invention are directed to one or more targets and thus can be used alone or in combination to eradicate bacteria. The invention relates to the delivery of toxic gene products or the combination of ribozymes and toxic gene products for the eradication of a pathogen or diseased cell. Specifically, the invention provides the delivery of one or more toxic proteins, antisense RNA, multi-ribozymes, or nucleic acids encoding the same, or a

combination thereof, to a cell, tissue, or subject containing an infectious bacteria or pathogen in order to eradicate such bacteria or pathogen.

5 The present invention further encompasses the use of a toxic agent and/or ribozymes of the present invention for the treatment of disease, viral infection, parasitic infection and microbial infection. The present invention further relates to a method of treating a subject having a proliferative disease of a specific tissue by inhibiting cell proliferation in the tissue, comprising administering to the subject a toxic agent and/or ribozyme operably linked to a tissue-specific promoter sequence, which promoter is specific for the diseased tissue, and whereby the ribozyme and/or toxic agent encoded by the nucleic acid is expressed, cell proliferation is inhibited, and the proliferative disease is treated.

10 The present invention further relates to a method of treating a subject having a pathogenic infection or disease, by inhibiting replication of the pathogen, comprising administering to the subject a toxic agent and/or ribozyme operably linked to a pathogen-specific promoter, whereby the ribozyme and/or toxic agent encoded by the nucleic acid is expressed, the pathogen is inhibited from replicating or is killed or rendered less fit, and the infection or disease is treated. In specific embodiments of the invention, the toxic agents of the invention are useful to treat microbial infections associated with severe burns, cystic fibrosis, cancer, or other immunocompromising conditions. The present invention encompasses the toxic agent(s) and/or ribozyme(s) of the present invention in pharmaceutical formulations.

20 The present invention further encompasses the use of the toxic agents and/or ribozymes of the present invention for research and screening purposes. In one embodiment of the present invention, the ribozymes and/or toxic agents may be used to screen for viral, microbial, prokaryotic, or eukaryotic gene products or molecules to be targeted in order to effectively inhibit the selected virus or microbial agent or selected cell.

25 In yet another embodiment, the present invention relates to a novel vector encoding the toxic agent(s) and/or ribozyme(s). The novel vectors of the present invention may be used to engineer a wide variety of toxic agents and/or ribozymes including, but not limited to, tissue-specific, pathogen-specific, promoter-specific, antimicrobial specific, antiviral specific, anticancer specific, antitumor specific, or target-specific.

30 In one embodiment, the invention relates to toxic agents which specifically target gene products essential for the survival or life cycle of a pathogen (such as replication, packaging, etc). In one embodiment, the present invention relates to naturally occurring bactericidal addiction system toxins which have been modified to be expressed in the absence of their corresponding addiction system antidote. In another embodiment, the present invention relates to naturally occurring addiction system toxins which have been

modified to be expressed at higher levels than their corresponding addiction system
antidote. In one example, an addiction system toxin (e.g., *doc*, *chpBK*, *kicB*, or *gef*) is used
as a toxic agent and is uncoupled from its antidote. In specific embodiments, the invention
provides for delivery of toxic agents such as bactericidal proteins (or nucleic acids encoding
5 such toxic agents) by a bacteriophage delivery system. In other specific embodiments, the
invention provides novel transfer plasmids encoding toxic agents which may be used in
combination with a bacteriophage delivery system in order to treat a bacterial infection in a
host.

The invention also relates to antisense RNA which targets essential
10 nucleotide sequences, such as *DicF1* or a *DicF1*-like antisense molecule that specifically
target a nucleotide sequence encoding a protein essential for replication or survival.
Further, the invention relates to modified antisense structures with increased stability which
act as lethal agents when expressed in bacteria. The invention also relates to toxic sense
molecules designed to target essential antisense molecules.

15 The present invention relates to multi-ribozymes and their use to target RNA
in a tissue-specific or pathogen-specific manner for the treatment of disease (such as
pathogen infection or cancer). The invention provides multi-ribozymes containing one or
more internal trans-acting ribozyme. Trans-acting ribozymes act in a target-specific manner
and therefore may act as a toxic agent to a pathogen (such as bacteria) or a selected cell
20 (such as a diseased cell). In accordance with the present invention, the multi-ribozyme may
comprise a) a trans-acting ribozyme flanked by 5' and 3' autocatalytically cleaving
ribozymes or enhanced autocatalytically cleaving ribozymes; b) a trans-acting ribozyme
flanked by either a 5' or 3' autocatalytically cleaving ribozyme; or c) multiple transacting
ribozymes, flanked by one or both 5' and 3' autocatalytically cleaving ribozymes or
25 enhanced autocatalytically cleaving ribozymes. Multi-ribozymes of the invention may also
be used to deliver one or more toxic agents to a pathogen cell or tissue. Ribozymes useful
in the present invention include those described in U.S. Patent 5,824,519 and PCT
publications No. WO98/24925, WO97/17433, WO98/24925, WO99/67400, which are
incorporated by reference herein in their entirety. In accordance with the present invention
30 the multi-transacting ribozymes may be targeted to the same site on the same RNA,
different sites on the same RNA or different RNAs. In accordance with the present
invention the multiple toxic agents may be targeted to the same site on the same target (such
as a cellular RNA or protein), different sites on the same target or different targets. For
example, in certain embodiments a toxic agent (such as an antisense nucleic acid or nucleic
35 acid encoding a toxic protein) may be engineered into a multi-ribozyme in place of a trans-

acting ribozyme, or in addition to a trans-acting ribozyme. In this embodiment, the toxic agent is flanked by a 5' and/or 3' autocatalytically cleaving ribozyme.

The invention additionally provides nucleic acids and expression cassettes which encode the toxic agent and/or ribozymes of the invention. These nucleic acids can be
5 used to express the toxic agent(s) and/or ribozyme(s) of the invention at the selected site.

At the molecular genetic level the coding sequence for a toxic agent, ribozyme, or multi-ribozyme of the invention may be placed under the control of one or more of the following genetic elements: a naturally occurring strong, intermediate, or weak constitutively expressed or regulated promoter from the targeted microorganism, or an
10 artificially contrived constitutively expressed or regulated promoter containing either a strong, intermediate or weak consensus sequence that accords the desired levels of ribozyme and/or toxic agent expression. The present invention relates to promoter elements which are pathogen-specific. The invention relates to promoter elements which are used to achieve pathogen-specific expression of the toxic agents of the present invention. The present
15 invention also relates to promoter elements which are tissue-specific. The invention relates to promoter elements which are used to achieve tissue-specific expression of the toxic agents of the present invention.

In one embodiment, the nucleic acids comprise a tissue-specific promoter operably linked to a sequence encoding one or more toxic agent(s). In another embodiment,
20 the nucleic acids comprise a pathogen-specific promoter operably linked a sequence encoding one or more toxic agent(s). In accordance with the present invention, toxic agents of the invention may act on the same or different targets.

The present invention relates to a toxic agent and/or a trans-acting ribozyme which targets any cellular, viral, bacterial, fungal, or other single cellular or multicellular
25 organism from any known taxonomic family, genus, or species. Another embodiment of the invention relates to a toxic agent which is lethal or toxic to a pathogen such as a bacteria, fungus, yeast, diseased cell.

The targets of the antimicrobial ribozyme therapeutics described herein are the RNAs of invading or normal flora microorganisms. The targets of the antimicrobial
30 toxic agent therapeutics described herein include RNAs, proteins, genes and other molecules of invading or normal flora microorganisms. The invention provides the delivery of a series of ribozymes and/or toxic agents directed towards essential, housekeeping, or virulence genes of one or a series of candidate microorganisms. Inactivation of essential proteins and virulence determinants render the invading microbes inactive or slow their
35 growth, while at the same time, the essential processes of the host are not significantly affected.

5 The present invention also relates to the delivery of the toxic agents of the invention to cell or pathogen by abiologic or biologic systems. In a specific embodiment, a toxic agent of the invention is delivered to a bacterial cell by a modified bacteriophage capable of infecting a pathogenic bacteria. In a further embodiment, bacteriophage are selected for their ability to infect a particular species or genera of bacteria, and are used to deliver a toxic agent for the eradication of such bacterial species or genera from a host. In a preferred embodiment, the delivery vehicle or nucleic acids native to the delivery vehicle are modified such that they contain insufficient genetic information for the delivery of nucleic acids native to the delivery vehicle. Thus, the modified delivery vehicle (*e.g.*, virion or bacteriophage) can serve as a molecular vehicle that delivers the ribozyme(s) and/or toxic agent(s) of the invention to the target cell or pathogen, but does not deliver replicable nucleic acids native to the delivery vehicle. Alternatively, an abiologic delivery system (*e.g.*, liposomes) can be used to package nucleic acid carrying the genetic elements necessary and sufficient for the proper expression of the ribozyme(s) and/or toxic agent(s).

10 In one embodiment, delivery of a toxic agent to a pathogen is by use of a bacteriophage or other delivery vehicle which targets the pathogen of interest. In one embodiment, a recombinant bacteriophage delivers the toxic agent or nucleic acids encoding the toxic agent to the pathogen.

The present invention provides compositions of matter which has resulted from the development of methods and compositions for the delivery of one or more ribozymes and/or toxic agents directed against fundamental and essential cellular processes specific to a targeted microorganism through an inactivated, altered, virus (virion), bacteriophage, or abiologic delivery vehicles, capable of delivering a nucleic acid comprising the toxic agent(s) and/or ribozyme(s) into the targeted microorganism. The microorganisms may be any virus, nonvirus, bacterium, or lower eukaryotes such as fungi, yeast, parasites, protozoa, or other eukaryotes that may be considered pathogens of humans, animals, fish, plants, or other forms of life. Thus, the invention has important implications in human and veterinary medicine.

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In certain preferred embodiments, a toxic agent of the invention is used as an antimicrobial therapeutic. A toxic agent may be used alone, or in combination with one or more other toxic agents. Thus, delivery of a toxic agent to an invading microorganism, kills or render it less fit. A toxic agent may also be used in combination with one or more ribozymes. Further, a combination of ribozymes and toxic agents may be used as an antimicrobial therapeutic.

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The toxic agent approaches of the invention offer advances for antimicrobial therapeutics including but not limited to: (1) the bypass of de novo or built-in drug

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resistance, which sophisticated microbes may have or develop (2) the decreased ability of cells to counteract ribozymes or toxic agents delivered into them, (3) the use of broad RNA targets and non-RNA targets available in microbes that can be attacked in simultaneously (4) the flexibility of custom design of the present delivery vehicle can be readily tailored to different families of organisms or different species of organisms, (5) the ease of assembly construction and manufacture of the modified delivery vehicle, (6) the availability of a variety of methods of administration of the pharmaceutical preparations of the invention such as topically, or via injection, inhalation, or ingestion, etc. (7) the ability to lyophilize the pharmaceutical preparation and thus confer stability to the antimicrobial therapeutic, (8) the reduced immunogenicity of the therapeutic preparations, and (9) the availability of animal test systems that enable the evaluation of the ribozymes and/or toxic agents of the invention. Therefore, the unique delivery approach and an aggressive mechanism for depriving the pathogen essential or important gene products can achieve the timely defeat of pathogen within the infected host. Accordingly, the invention has important implication in the eradication of drug-resistant pathogens.

4. BRIEF DESCRIPTION OF THE FIGURES

- Figure 1A Diagram depicts the components of the *lacI*-regulated broad spectrum promoter.
- Figure 1B The sequence of the LEASHI promoter (SEQ ID NO:1).
- Figure 1C The sequence of a modified *rrnB* promoter (SEQ ID NO:2).
- Figure 1D The sequence of the *Anr* promoter (SEQ ID NO:3).
- Figure 1E The sequence of the *Proc* promoter (SEQ ID NO:4).
- Figure 1F The sequence of the *Arc* promoter (SEQ ID NO:5).
- Figure 1G The sequence of the *TSST-1* promoter (SEQ ID NO:6).
- Figure 2 Diagram of a β -lactamase reporter plasmid.
- Figure 3A-B Expression vectors for cloning Toxic Agents.
- Figure 4 Assay for lethality of Toxic Agents
- Figure 5 Growth of *E. coli* harboring a *doc* expression plasmid.
- Figure 6A-B Structure of a Transfer Plasmid.
- Figure 7 Delivery Efficiency of the Transfer Plasmid by the P1 bacteriophage vehicle to *E. coli*.
- Figure 8 Scheme for generation of the P1 *pac* site knockout.

- Figure 9 Identification and confirmation of the P1 *pac* site knockout by PCR screening.
- Figure 10 Diagram of the *pacABC* Complementing plasmid.
- Figure 11 Recombination between the P1 *pac* mutant and the *pacABC* Complementing plasmid.
- Figure 12 Sequence of the minimal P1 *pac* site (SEQ IS NO: ~~8~~⁸).
- Figure 13 Immunogenicity of replicating phage in mice.
- Figure 14 Comparison of original and long-circulating P1 phage persistence *in vivo*.
- Figure 15 Treatment of *P. aeruginosa* (PA01) infections in embryonated hen eggs.
- Figure 16 In vitro killing of *E. coli* EC-4 bacterial cells.
- Figure 17 Treatment of *E. coli* EC-4 infection in embryonated hen eggs.
- Figure 18 Sequence of the *DicF1* molecule (SEQ ID NO: ~~8~~⁸). (SEQ ID NO: 10)
- Figure 19 Diagram and nucleotide sequence of the pClip ribozyme cassette. (SEQ ID NO: 11)
- Figure 20 Diagram and nucleotide sequence of the pChop ribozyme cassette.
- Figure 21 Schematic diagram of the pSnip ribozyme cassette. pSnip includes sequences of the pClip triple ribozyme cassette, catalytic core targeted ribozymes comprising two linked trans-acting ribozymes, and sequences from the pChop triple ribozyme cassette.
- Figure 22A A schematic of DNA encoding the ribozyme used in the molecular sequence of events in ribozyme maturation and action.
- Figure 22B The primary RNA transcript. Autocatalytic cleavage takes place upon completion of transcription.
- Figure 22C The release of the trans-acting ribozyme. As a direct result of cleavage of the two cis-acting ribozymes, the internal ribozyme containing a reverse and complementary sequence to the mRNA target is released.
- Figure 22D The sequence specific hybridization of the ribozyme. The internal or trans-acting ribozymes comprise two trans-acting ribozymes linked by a short nucleotide "spacer". Each of the two trans-acting ribozymes contain a sequence that is reverse complementary to the targeted message of the same or at different sites. The ribozyme is synthesized at a concentration sufficient to locate and hybridize to all or substantially all targeted transcripts.
- Figure 22E The trans-catalytic cleavage. Upon hybridization of the internal trans-acting ribozyme to the targeted mRNA transcript, the internal ribozyme achieves a

catalytic topology and cleaves the targeted message. Upon cleavage the trans-acting ribozyme is released and its activity and function are recycled.

5. DETAILED DESCRIPTION OF THE INVENTION

5 The present invention provides toxic agent(s) and/or ribozyme(s) and their use in a tissue-specific, target-specific, or pathogen-specific manner for the treatment of disorders and disease related to bacterial, parasitic or viral infections or to cellular proliferation, and cancers. The ribozymes and/or toxic agents of the present invention may be engineered to target one or more specific RNAs contained in a specific cell or tissue in
10 the host. The ribozymes of the present invention may also be engineered to target one or more specific RNAs encoded by a specific pathogen, virus, or microbial agent. The toxic agents of the present invention may also be engineered to target one or more specific RNAs, proteins, or molecules of a specific pathogen, virus, or microbial agent.

 The present invention also provides toxic agents which are lethal or toxic to
15 a selected pathogen. In one embodiment of the invention, the toxic agents of the invention comprise toxic proteins which cause lethality to a pathogen or selected cell (*e.g.*, a diseased cell) or which render the pathogen or selected cell less fit. In one embodiment, such toxic proteins of the invention are lethal when overexpressed in a pathogen or selected cell. In other embodiments, a toxic protein is an exogenous protein that is toxic when expressed in a
20 pathogen or selected cell. A toxic protein of the invention may further be engineered to have increased toxicity. For example, many methods are known in the art for introducing mutations, deletion, insertions etc. into a known sequence. Thus, optimization of a toxic protein is provided. The invention also provides methods for inhibiting the toxicity of a toxic protein, so that the toxic protein may be produced or manufactured in a producing cell.
25 Inhibiting the toxicity may be performed by any methods known in the art, for example, the toxic protein may be expressed from an inducible promoter which allows expression to be turned on/off under appropriate conditions. A toxic protein may be expressed in a cell without causing lethality in the cell by overexpressing an antidote protein in the same cell. Other methods will be apparent to one skilled in the art and are within the scope of the
30 invention.

 The present invention provides toxic agents and methods for specifically targeting toxic agents to bacteria or bacteria-infected cells or other pathogens. Toxic agents of the present invention are directed to one or more targets and thus can be used alone or in combination to eradicate bacteria. Specifically, the invention provides the delivery of one
35 or more toxic proteins, antisense RNAs, multi-ribozymes, or nucleic acids encoding the

same, or a combination thereof, to a cell, tissue, or subject containing an infectious bacteria or pathogen in order to eradicate such bacteria or pathogen.

The present invention further encompasses the use of the toxic agents and/or ribozymes of the present invention as therapeutics and pharmaceutical compositions. In
5 specific embodiments of the invention, the toxic agents of the invention are useful to treat microbial infections associated with severe burns, cystic fibrosis, cancer, or other immunocompromising conditions.

The present invention further encompasses the use of the toxic agents and/or ribozymes of the present invention for research and screening purposes. In one embodiment
10 of the present invention, the ribozymes and/or toxic agents may be used to screen for viral, microbial, prokaryotic, or eukaryotic gene products or molecules to be targeted in order to effectively inhibit the selected virus or microbial agent or selected cell.

5.1. PATHOGEN-SPECIFIC AND TISSUE-SPECIFIC TOXIC AGENTS

The invention provides specific nucleic acids which act as or encode toxic
15 agents and are therefore useful as antimicrobial agents. A variety of toxic agents are within the scope of the invention. For clarity, the toxic agents of the invention are described herein below in several sub-types. The toxic agents of the invention include but are not limited to antisense nucleic acids, toxic gene products, sense nucleic acids.

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5.1.1. TOXIC GENE PRODUCTS

The present invention relates to the use of toxic gene products or toxic proteins as toxic agents for the treatment of disorders and disease related to bacterial, parasitic, fungal, or viral infections or to cellular proliferation, and cancers, or to diseased
25 cells. A toxic gene product of the invention is any gene product (such as DNA, RNA or protein), which is toxic to a pathogen or selected cell (such as a diseased cell). Such toxic gene products may be naturally occurring (endogenous), or may be non-naturally occurring (exogenous) in the target pathogen or selected cell. A toxic agent of the invention may be a chromosomally encoded, plasmid encoded, pathogen encoded, synthetic, or encoded in any
30 other nucleic acid or nucleotide sequence. The present invention provides toxic agents which are endogenous toxic gene products that are expressed in a pathogen or selected cell which kill or render the pathogen or selected cell less fit. The present invention also provides toxic agents which are exogenous toxic gene products that are introduced into or expressed in a pathogen or selected cell which kill or render the pathogen or selected cell
35 less fit. A pathogen or selected cell which is less fit is one which is weakened, or which is more susceptible to chemical treatment (such as drugs, toxins, pharmaceuticals, mutagens,

solvents, etc.), or which is more susceptible to physical stress (such as temperature), or which is more susceptible to genetic alterations (such as by radiation or UV), or is more susceptible to environmental changes (such as available nutrients).

In several embodiments, the present invention provides the use of a plasmid addition system protein as a toxic agent when expressed in bacteria or a selected cell. For example, in certain types of bacteriophage, the lysogenic (dormant) pathway is manifested by a bacterial cell maintaining only a single copy of the bacteriophage DNA in the form of a plasmid. In order to assure that both daughter bacterial cells receive a copy of the plasmid, a "plasmid addition system" or "post-segregation system" is used by the cells which ensures that only bacterial cells which receive a copy of the plasmid will survive.

In one embodiment of the invention, a post-segregation system or plasmid addition system toxin, is used as a toxic agent to a pathogen (such as bacteria) by overexpression of the toxin. Such overexpression of the toxin uncouples the toxin and the antidote, leading to toxicity, and preferably lethality, in the cell containing the overexpressed toxic agent.

For example, in one embodiment, the invention provides toxic agents which specifically target gene products essential for the survival or life cycle of a pathogen (such as replication, packaging etc). In one embodiment, the present invention provides naturally occurring addition system toxins which have been modified to be expressed in the absence of the addition system antidote. In another embodiment, the present invention provides naturally occurring addition system toxins which have been modified to be expressed at higher levels than the addition system antidote. In one example, an addition system toxin (e.g., *doc*, *chpBK*, *kicB*, or *gef*) is used as a toxic agent and is uncoupled from its antidote. In another embodiment of the invention, a chromosomally encoded toxic gene product (such as *chpBK*, *kicB*, or *gef*) is used as a toxic agent to a pathogen by overexpression of the toxic gene product.

In certain embodiments, toxic agents include but are not limited to Shiga-like toxins of *E. coli*, cholera toxin of *Vibrio cholerae*, and cytotoxins of *P. aeruginosa*. For example, phage K139 confers to *V. cholera* a gene product that enhances enzymatic activity of cholera toxin. Such toxins are within the scope of the invention and may be used as a toxic agent in association with the methods and compositions of the invention.. In certain embodiments of the invention, the bacteriocidal toxic agent is derived from a bacterium including but not limited to *Staphylococcus aureus*, *Enterococcus faecalis*, or *Pseudomonas aeruginosa*.

In another embodiment, the antidote of a toxin is the target of a trans-acting ribozyme or toxic agent of the invention. Thus, when the antidote is inactivated by the

trans-acting ribozyme or toxic agent, the toxin is no longer neutralized or inactivated by the antidote, thus leading to toxicity, and preferably lethality to the pathogen.

In yet another embodiment, when the antidote is itself an antisense RNA, a sense RNA may be synthesized as a toxic agent and delivered to inactivate the antisense antidote. Thus, when the antidote is inactivated by the sense RNA, the antidote is no longer available to inactivate the toxin, thus leading to toxicity, and preferably lethality.

One example of an addiction system toxin that may be used in connection with the invention is *doc* (death on curing; Lehnher H, et al., 1993, J. Mol. Biol. 233:414-28). The protein encoded by *doc* is lethal or toxic in both Gram-negative and Gram-positive organisms (e.g., *E. coli*, *P. aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*). *doc* acts as a bacterial cell toxin to which *Phd* (prevention of host death) is the antidote. Accordingly, the invention provides for plasmids expressing *doc* which can be delivered to a bacterial pathogen in order to render the pathogen less fit, and preferably eradicate the pathogen. A particular advantage of *doc* is that *doc* has little to no toxicity to eukaryotic cells, and thus may be administered safely to a eukaryotic host.

Specific examples of addiction system toxins or chromosomally encoded toxins, or other toxic agents which may be used in connection with the invention include but are not limited to *ccdB*, *kid*, *perK*, *parE*, *doc*, *higB*, *chpAK*, *chpBK*, *kicB*, *hoc*, *srnB*, *flmA*, *pmdA*, *relF*, *gef*, *kilA*, *kilB*, *kilC*, *kilE*, *traL*, *traE*, *sigB*, *hok*, *pemK*, *lysostaphin*, and *kikA*. Examples of antidotes which may be used as in the methods of the invention include but are not limited to *ccdA*, *kis*, *pemI*, *parD*, *phd*, *higA*, *chpAI*, *chpBI*, *kicA*, *soc*, *srnC*, *flmB*, *pndB*, *sof*, *korA*, *korB*, *korC*, *korD*, *korE*, and *korF*. Thus, the invention herein provides a method of using a an addiction system toxin (such as *doc*) or other toxic protein, as a toxic agent of the invention. The invention also provides methods for inhibiting or inactivating antidotes of a toxin. The invention further provide co-expression of a toxin and its corresponding antidote for manufacturing purposes.

In certain specific embodiments, the invention provides toxic agents *chpBK*, *kicB*, and *gef*. Each of the proteins of *kicB*, or *gef* are lethal in *E. coli* but not in *P. aeruginosa*. Accordingly, the invention provides for the use of *kicB* or *gef* in the eradication or treatment of bacterial infections of *E. coli*. In one embodiment, *kicB* or *gef* encoding nucleic acids are delivered by a to the *E. coli* by a P1 bacteriophage of the invention containing a transfer plasmid, said transfer plasmid encoding the *kicB* or *gef* toxic agents (or both).

In another specific embodiment of the invention, the *chpBK* protein is a toxic agent of the invention and is lethal to *E. coli* and toxic or lethal in *P. aeruginosa*. Accordingly, the invention provides for the use of *chpBK* in the eradication or treatment of

infections of *E. coli* or *P. aeruginosa*. In one embodiment, *chpBK* nucleic acids are delivered by a to *E. coli* or *P. aeruginosa* by a P1 bacteriophage of the invention, containing a transfer plasmid, said transfer plasmid encoding the *chpBK* toxic agent. The antidote protein that antagonizes *chpBK* function is called *ChpBl*. Accordingly, the
5 invention provides, for the co-expression of the antidote *ChpBl* and *chpBK* for manufacturing purposes.

In several embodiments of the invention, the toxic gene, such as *doc*, *chpBK*, *kicB*, or *gef*, is placed under the control of an inducible promoter and is uncoupled from the antidote. In one embodiment, the promoter is the P1 lytic promoter P53. In a preferred
10 embodiment, the promoter is the LEASHI promoter. In a preferred embodiment, for the treatment of *P. aeruginosa* infections, the invention provides *P. aeruginosa* specific promoters, *anr*, *arc* or *proC*.

In other specific embodiments of the invention, a consensus ribosome binding site (GGAGGTGXXXXATG, ^(SEQ ID NO: 12) wherein X is any nucleotide) may be inserted
15 immediately upstream of the nucleic acids encoding the toxic agent and leads to increased expression of the toxic agent. The provides for the use of a combination of a promoter and a ribosome entry site(s) to modulate expression of a toxic agent or ribozyme.

It is also within the scope of the invention that more than one toxic agent may be used to eradicate or treat an infection. For example, it is contemplated that two or more
20 toxic agents may be engineered into a single transfer plasmid for delivery by a bacteriophage. Such bacteriophage could serve to deliver nucleic acids encoding multiple toxic agents to target bacteria. Alternatively, two or more transfer plasmids may be carried by a single bacteriophage, wherein each transfer plasmid encodes different toxic agents. In this embodiment, when more than one Transfer plasmids are used, such plasmids are
25 designed such that the two or more plamids are non-recombinogenic. Such methods of engineering non-recombinogenic sequences are known in the art. Additionally, in this embodiment, the two or more engineered plasmids will preferably have different origins of replication. In this manner, the bacteriophage serves to deliver nucleic acids encoding multiple toxic agents. In yet a third alternative, bacteriophage may be designed to carry
30 multiple toxic agents on multiple transfer plasmids. When two or more toxic agents are encoded within a single bacteriophage, the nucleic acids encoding such toxic agents may be operably linked to the same promoter, or different promoters (e.g., see sections 5.4 and 5.4.1 herein).

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5.1.2. ANTISENSE

The invention provides specific nucleic acids which act as toxic agents and are therefore useful as antimicrobial agents. The invention provides antisense RNA molecules which target an RNA of a pathogen or selected cell. Target RNAs of the invention may be pathogen-specific RNAs, tissue-specific cellular RNAs, or disease-specific RNAs. The invention also provides modified and enhanced antisense nucleic acids which target pathogen-specific RNAs, tissue-specific cellular RNAs, or disease-specific RNAs.

The proposed target of the toxic antisense molecule of the invention is the RNA of a gene which plays a critical role in the survival of the pathogen, or which is essential to the pathogen's life cycle. The present invention also encompasses modifications to naturally occurring antisense molecules which modulate the expression of an essential gene product of a pathogen. For example, as described below, one proposed target of an antisense of the invention is the *ftsZ* gene whose gene product plays a critical role in the initiation of cell division of *E. coli*.

In another embodiment, the toxic agents of the invention comprise antisense molecules designed to have enhanced inhibition of target RNAs. The toxic agents which comprise antisense molecules of the invention are engineered to more specifically bind target RNAs in that the sequences of such toxic antisense molecules are designed to have increased complementarity to a target sequence such as an essential RNA of a pathogen or selected cell. Such toxic antisense molecules are therefore more specific to their targets and hence, have increased efficacy. The invention provides antisense toxic agents and ribozymes which are also modified with a hairpin structure to create a more stable molecule. The antisense toxic agents of the invention may also be expressed to a high level in a target pathogen or cell by any method known or cell by any method known in the art. For example, an antisense toxic agent may be expressed *in trans* from a multi-copy expression plasmid using a strong regulatable promoter. The antisense toxic agent may also be operably linked to a tissue-specific or pathogen-specific promoter such that the antisense molecule is only expressed in a pathogen or cell which uses the same promoter.

Specifically, the invention provides antisense RNAs which target essential nucleotide sequences, such as *DicF1* or a *DicF1*-like antisense molecule that specifically target a nucleotide sequence which encodes a protein essential for replication or survival. Further, the invention provides modified antisense structures with increased stability to act as lethal agents when expressed in bacteria. The invention also provides toxic sense molecules designed to target essential antisense molecules.

In another embodiment of the invention the toxic agents comprise sense RNA molecules targeted to antisense RNAs which are required for the survival of the pathogen or cell. For example, an antidote of a toxic protein (such as an addiction system toxin) may be in the form of an antisense molecule which regulates the expression of the toxin. Such an antisense antidote allows the pathogen or cell to survive in the presence of such toxin. The invention provides inhibition of the antisense antidote by a toxic agent in the form of a sense RNA molecule.

In certain embodiments a combination of two or more toxic molecules may be delivered to a pathogen (such as *E. coli*, *P. aeruginosa*, etc.) in order to cause lethality. In this embodiment, the toxic antisense may be directed to the same target, or different targets. When different targets of a pathogen or cell are targeted, such targets may be involved in the same biological pathway within the pathogen or different biological pathways.

In a specific embodiment, the antisense sequence is based on *DicF* (Bouche F, et al., 1989, Mol Microbiol. 3:991-4). Such modified *DicF* sequence is referred to as *DicF1* (SEQ ID NO:8). Naturally occurring *DicF* is part of an intercistronic region that when expressed in *Escherichia coli* causes inhibition of cell division. This inhibition does not require the translation of *DicF* mRNA into protein, instead, *DicF* RNA exerts its inhibitory effect as an antisense molecule.

The proposed target of *DicF* is the *ftsZ* gene whose gene product plays a critical role in the initiation of cell division of *E. coli*. Temperature sensitive mutations of the *ftsZ* gene indicate that it is essential for viability of *E. coli*. Without limitation as to mechanism, *DicF* RNA is believed to bind specifically to the 5' untranslated region of *ftsZ* mRNA, thereby inhibiting *ftsZ* protein expression. Cells lacking the *ftsZ* protein are unable to divide and ultimately die. *DicF* homologs have been identified in a variety of other bacteria although it is not known whether they exert a similar function.

The present invention provides for modified *DicF* nucleic acids, called *DicF1* or *DicF1*-like RNAs, which are used as antimicrobial agents, or toxic agents of the invention. *DicF1* RNA is a superior antisense molecule as compared to the endogenous *DicF* RNA. It has been modified by increasing its complementarity to the *ftsZ* 5' untranslated mRNA. It is therefore more specific to its target and hence, has increased efficacy. An auto hairpin structure has further been enhanced to create a more stable molecule. The invention also provides modifications of other naturally occurring antisense molecules, such as nucleotide sequences which have similar functions as *DicF* in modulating the expression of gene products essential to the pathogen's life cycle or survival. Such nucleic acid is referred to as a *DicF1*-like nucleic acid. In contrast to the

endogenous *DicF*, the *DicF1* or a *DicF1*-like nucleic acid of the invention may be expressed *in trans* from a multi-copy expression plasmid. Further, the *DicF1* or *DicF1*-like nucleic acids may be operably linked to a variety of promoters that may be used to control the strength, timing, or distribution of such expression. *DicF1* or a *DicF1*-like nucleic acid may also be expressed *in trans* from a ribozyme cassette. The combination of these features results in *DicF1* or *DicF1*-like nucleic acid being an effective antimicrobial agent against a pathogen (such as *E. coli*). In other embodiments, modifications to the sequence of an antisense of the invention allows targeting against a variety of other bacteria. In other embodiments, modifications to the sequence of an antisense of the invention allows targeting in a pathogen-specific manner. The invention also provides *DicF1*-like nucleic acids which may be used as toxic agents in bacteria, bacteria-infected cells, or other pathogens which have complementary RNA targets.

5.1.3. RIBOZYMES

The present invention provides methods by which a trans-acting ribozyme may be used in addition to the toxic agents of the invention. Further, a multi-ribozyme may be used as an expression system for one or more toxic agents or trans-acting ribozymes. These ribozymes of the invention can be used, for example, to destroy tissue-specific disease, or to treat bacterial, viral, or parasitic infections. The ribozymes of the present invention may comprise one or more multi-ribozymes.

In accordance with the present invention, the multi-ribozyme may comprise one or more ribozymes or one or more ribozyme cassettes. Each cassette in turn may consist of a catalytic core (*e.g.*, containing one or more trans-acting ribozymes or containing one or more toxic agents) and one or more flanking regions. The catalytic core can target a pathogen, by specifically inhibiting a pathogen-specific target. The catalytic core can target a cell (such as a diseased cell), by specifically inhibiting a tissue-specific target (such as disease-specific target). Further, as described in sections below, the multi-ribozymes of the invention also provide a means of delivering toxic agents to a cell, and expressing toxic agents of the invention (including antisense RNA, toxic gene products) in a cell or tissue-specific, or pathogen-specific manner. In one embodiment, the ribozyme cassette may consist of a 5' autocatalytically cleaving ribozyme sequence, a core catalytic ribozyme comprising a trans-acting ribozyme and a 3' autocatalytically cleaving ribozyme. In another embodiment, the multi-ribozymes comprise a cassette including, the enhanced 5' and 3' autocatalytically cleaving ribozyme sequence. In another embodiment, the multi-ribozymes contain one or more internal trans-acting ribozymes. Such trans-acting ribozymes may be directed to the same site on the same RNA, different sites on the same RNA, or different

RNAs. Thus, trans-acting ribozymes of the invention may target a pathogen-specific RNA or tissue-specific RNA.

The present invention also provides multi-ribozymes and their use to target RNA in a tissue-specific or pathogen-specific manner for the treatment of disease such as bacterial infection. The invention provides multi-ribozymes containing one or more internal trans-acting ribozyme. Trans-acting ribozymes act in a target-specific manner and therefore may, in certain embodiments, act on a pathogen (such as bacteria) or a selected cell (such as a diseased cell) to enhance the use of toxic agent. In accordance with the present invention, the multi-ribozymes may comprise a) a trans-acting ribozyme or toxic agent flanked by 5' and 3' autocatalytically cleaving ribozymes or enhanced autocatalytically cleaving ribozymes; b) a trans-acting ribozyme or toxic agent flanked by either a 5' or 3' autocatalytically cleaving ribozyme; or c) multi-transacting ribozymes and/or multiple toxic agents, flanked by one or both 5' and 3' autocatalytically cleaving ribozymes or enhanced autocatalytically cleaving ribozymes. For example, in a specific embodiment, the invention provides a multi-ribozyme with two trans-acting ribozymes, wherein the first trans-acting ribozyme cleaves an HBV target, and the second trans-acting ribozyme cleaves a HCV target. In this embodiment, it may also be desirable to target the expression of such multi-ribozyme to the liver, *e.g.*, by operative association with a liver-specific promoter. Thus, the multi-ribozymes of the invention may be used to deliver one or more toxic agents to a bacteria or bacteria-infected cell or tissue. In accordance with the present invention the multi-transacting ribozymes may be targeted to the same site on the same RNA, different sites on the same RNA or different RNAs. In accordance with the present invention the multiple toxic agents may be targeted to the same site on the same target (such as a cellular RNA or protein), different sites on the same target or different targets.

The ribozymes of the present invention possesses sufficient catalytic activity to inactivate the RNA of the targeted RNAs. From an antimicrobial perspective, hammerhead-type ribozymes are especially attractive since the molecule inactivates gene expression catalytically through the cleavage of the phosphodiester bond of the mRNA. Furthermore, hammerhead-type ribozymes have been re-engineered to function in an intermolecular or transducer (trans) acting state (Haseloff et al., 1988, Nature 334(6183):585-91; Uhlenbeck. O.C., 1987, Nature 328(6131):59). The catalytic activity of the ribozyme requires a sufficient concentration of the divalent cation, Mg^{+2} , and substrate. The substrate can have any sequence as long as the cleavages site contains the recognition element NUX, where N represents any nucleotide, U corresponds to uracil, and X is any nucleotide except G (Koizumi et al., 1989, Nucleic Acids Resonant. 17(17):7059-71) .

Ribozymes have been widely demonstrated to function *in vivo* (Christoffersen et al., 1995, J. Med. Chem. 38(12):2023-37; Inokuchi et al., 1994, J. Biol. Chem. 269(15):11361-6) . The present invention improves the initial design of hammerhead-type ribozymes (Taira et al., 1991, NAR 19(9):5125-5130) by constructing multi-ribozymes consisting of ribozyme
5 cassettes. Ribozyme cassettes contain one or more cis-acting hammerhead ribozymes flanking a ribozyme that inactivates the targeted RNA(s) as well as one or more flanking sequences. Upon transcription the targeted ribozyme is released as a 60-70 base transcript which not only improves its specificity by reducing non-specific interactions but also improves its catalytic activity as well. This invention includes modifications to and use of
10 the ribozyme described in U.S. Patent 5,824,519 and PCT publications No.WO98/24925, WO97/17433, WO98/24925, WO99/67400, which are incorporated by reference herein in their entirety.

5.2. NUCLEIC ACIDS ENCODING TOXIC AGENTS OR RIBOZYMES

15 The invention also provides nucleic acids and expression cassettes which encode the ribozymes and/or toxic agents of the invention. These nucleic acids can be used to express the ribozymes or toxic agents of the invention at the selected site. The site can be tissue-specific in the case of treating tissue-specific cancers or disease, or it can be pathogen-specific in the case of ribozymes or toxic agents that prevent replication of
20 infectious agents to treat infection (*e.g.*, hepatitis, herpes, malaria, tuberculosis, bacterial infections etc.). The invention provides nucleic acids which encode toxic agent(s) and/or ribozyme(s) which are target-specific. The invention also provides nucleic acids which encode toxic agent(s) and/or ribozyme(s) operably linked to a tissue-specific or pathogen-specific promoter.

25 There are several options for constructing the multi-ribozyme encoding sequences: 1) ribozymes directed to different targets in the same pathogen 2) multiple copies of the same ribozyme 3) multiple ribozymes directed to multiple targets, and 4) multiple ribozymes directed to different sites on the same target. There are also several options for constructing the toxic agent encoding sequences: 1) toxic agents directed to
30 different targets in the same pathogen 2) multiple copies of the same toxic agent 3) multiple toxic agents directed to multiple targets, and multiple toxic agents directed to the same target. Further, toxic agents and ribozymes may be combined in various ways, *e.g.*, a multi-ribozyme and a nucleic acid encoding a toxic agent may be engineered in a single construct under one promoter. The promoter can have the chosen level of specificity as described
35 herein.

5 The nucleic acids of the invention encode one or more toxic agents of the invention. Thus, nucleic acids encoding toxic proteins of the invention include but are not limited to addiction system toxins. The invention further provides modified and enhanced addiction system toxins which have been engineered to be more toxic or more specific to a particular target pathogen. The present invention provides nucleic acids encoding antisense molecules targeted to RNA of a gene which plays a critical role in the survival of the pathogen, or which is essential to the pathogen's life cycle. The present invention also encompasses nucleic acids comprising modifications to naturally occurring antisense molecules which modulate the expression of an essential gene product of a pathogen.

10 The nucleic acids of the invention also relate to those encoding antisense molecules of the invention. The invention provides modified and enhanced antisense molecules which have enhanced stability, enhanced complementarity to a target RNA, or enhanced specificity for a target RNA or target pathogen. The invention also provides nucleic acids encoding modified naturally occurring antisense molecules, such as nucleotide
15 sequences which have similar functions as *DicF* in modulating the expression of gene products essential to the pathogen's life cycle or survival.

The nucleic acids of the invention also relate to nucleic acids encoding sense RNA molecules capable of targeting an essential antisense molecule.

20 The nucleic acid, encoding a toxic agent selected from the group consisting of *ccdB*, *kid*, *perK*, *parE*, *doc*, *higB*, *chpAK*, *chpBK*, *kicB*, *hoc*, *srnB*, *flmA*, *pmdA*, *relF*, *gef*, *kilA*, *kilB*, *kilC*, *kilE*, *traL*, *traE*, *sigB*, *hok*, *pemK*, *lysostaphin*, and *kikA* is provided. The nucleic acid encoding the toxic agent *DicF1*, or *DicF1*-like, is provided.

30 In several embodiments, nucleic acids of the invention encode a catalytic multi-ribozyme(s) that contains two separable functional regions including a) a catalytic sequence (also known as the "catalytic core") which cleaves the target RNA, and b) flanking regions which include cis-acting autocatalytically cleaving ribozyme(s). As described above, the catalytic core consists of one or more trans-acting ribozyme(s) and/or one or more toxic agent(s). The present invention provides nucleic acid which encode an internal targeted ribozyme containing two or more trans-acting ribozymes, wherein each of the
35 separate trans-acting ribozymes can be targeted to the same or different target RNA molecules. By nucleic acid complementarity, the binding site directs the ribozyme core to cleave a specific site on the target RNA molecule. The length of flanking sequences have implications not only for specificity, but also for the cleavage efficiency of the individual ribozyme molecules. In the present catalytic ribozyme, the flanking sequences are highly specific for the target RNA; yet allow ready dissociation from the target RNA once cleavage occurs. This permits cycling of the ribozyme and reduces the amount of ribozyme required

to be effective. A range of binding/dissociation values from 16-21 Kcal is expected to be effective. The present invention provides nucleic acid which encode a two or more toxic agents, wherein each of the toxic agents can be targeted to the same or different target molecules.

5 The invention additionally provides nucleic acids and expression cassettes which encode the toxic agent and/or ribozymes of the invention. These nucleic acids can be used to express the toxic agent and/or ribozyme of the invention at the selected site. In one embodiment, the nucleic acid comprise a tissue-specific promoter operably linked to a toxic agent. In another embodiment, the nucleic acids and expression cassettes of the invention
10 comprise a tissue-specific promoter operably linked to a sequence encoding a catalytic ribozyme comprising one or more target RNA-specific trans-acting ribozymes and one or more toxic agents. In another embodiment, the nucleic acids comprise a pathogen-specific promoter from a sequence encoding a toxic agent. In another embodiment, the nucleic acids and expression cassettes of the invention comprise a pathogen-specific promoter operably
15 linked to a sequence encoding a 5' autocatalytically cleaving ribozyme sequence, a catalytic ribozyme comprising one or more target RNA-specific trans-acting ribozymes and/or pathogen-specific toxic agents, and a 3' autocatalytically cleaving ribozyme sequence. In accordance with the present invention, the expression cassettes may be engineered to express two or more multi-ribozymes containing trans-acting ribozymes which act on the
20 same or different targets. The expression cassettes may also be engineered to express two or more multi-ribozymes containing 5' and 3' autocatalytically cleaving ribozymes with either slow or enhanced cleavage activity.

 The expression cassettes of the invention or the nucleic acids encoding the toxic agents of the invention may be placed into any suitable plasmid known in the art (such
25 as a bacteriophage transfer plasmid, bacterial plasmid, or eukaryotic expression plasmid). The invention also provides novel and modified plasmids for use in accordance with the invention.

 At the molecular genetic level the coding sequence for a toxic agent, ribozyme, or multi-ribozyme of the invention may be placed under the control of one or
30 more of the following genetic elements: a naturally occurring strong, intermediate, or weak constitutively expressed or regulated promoter from the targeted microorganism, or an artificially contrived constitutively expressed or regulated promoter containing either a strong, intermediate or weak consensus sequence that accords the desired levels of ribozyme and/or toxic agent expression. The present invention provides promoter elements which are
35 pathogen-specific. The invention provides promoter elements which are used to achieve pathogen-specific expression of the toxic agents of the present invention. The present

invention provides promoter elements which are tissue-specific. The invention provides promoter elements which are used to achieve tissue-specific expression of the toxic agents of the present invention. Accordingly, the present invention provides nucleic acids encoding promoter elements which are pathogen-specific. The invention provides promoter elements
5 which are used to achieve pathogen-specific expression of the toxic agent(s) and/or ribozyme(s) of the present invention. The present invention provides promoter elements which are tissue-specific. The invention provides promoter elements which are used to achieve tissue-specific expression of the toxic agent(s) and/or ribozyme(s) of the present invention.

10 In one embodiment, the nucleic acid comprise a tissue-specific promoter operably linked to a sequence encoding one or more toxic agent(s). In another embodiment, the nucleic acids comprise a tissue-specific or pathogen-specific promoter operably linked to a sequence encoding at least one autocatalytic ribozyme and one or more trans-acting ribozymes. In another embodiment, the nucleic acids comprise a tissue-specific or
15 pathogen-specific promoter operably linked to a sequence encoding at least one or more toxic agents. In another embodiment, the nucleic acids comprise a pathogen-specific promoter operably linked to a sequence encoding at least one autocatalytic ribozyme and one or more trans-acting ribozymes and one or more toxic agents. In accordance with the present invention, the trans-acting ribozymes and/or toxic agents of the invention may act
20 on the same or different targets.

In yet another embodiment, the present invention provides a novel vector or plasmids encoding the toxic agent(s) and/or ribozyme(s) of the invention. The novel vectors of the present invention may be used to engineer a wide variety of toxic agents and/or ribozymes including, but not limited to, tissue-specific, pathogen-specific, promoter-
25 specific, antimicrobial specific, antiviral specific, anticancer specific, antitumor specific, or target-specific. The invention also relates to a vector or plasmid origin of replication which modulates specificity of the replication of a vector or plasmid in a cell or pathogen. The invention also relates to the copy number of a vector or plasmid in a selected cell or pathogen to modulate the dose of the toxic agent and/or ribozyme.

30 In a specific embodiment, the invention provides novel plasmids which encode a toxic protein. In another specific embodiment, the invention provides novel plasmids which encode a mutant bacteriophage *pac* site or a mutant bacteriophage *pacABC* sequence.

5.2.1. EUCARYOTIC AND PROCARYOTIC EXPRESSION VECTORS

The present invention encompasses expression systems, both eucaryotic and procaryotic expression vectors, which may be used to express the toxic agents and/or multi-ribozymes of the invention. The DNA expression vectors and viral vectors containing the nucleic acids encoding the toxic agents of the present invention may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the expression vectors and viral vectors of the invention by expressing nucleic acid encoding a toxic agent and/or multi-ribozyme sequences are described herein.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing gene product coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, *supra*, and Ausubel et al., 1989, *supra*. Alternatively, nucleic acids capable of encoding a toxic agent and/or ribozyme sequence may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

A variety of host-expression vector systems may be utilized to express the selected toxic agent and/or multi-ribozyme of the invention. Such host-expression systems represent vehicles by which the sequences encoding the toxic agents or ribozymes of the invention may be introduced into cells, tissues, or pathogens both *in vivo* and *in vitro* but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, to express a toxic agent and/or ribozymes of the invention. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing selected toxic agent(s) and/or multi-ribozyme coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the selected toxic agent(s) and/or multi-ribozyme coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the selected toxic agent(s) and/or multi-ribozyme coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing selected toxic agent(s) and/or multi-ribozyme coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells

(e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

5.3. DELIVERY AND EXPRESSION OF TOXIC AGENTS

5 The invention also provides a novel vehicle for the delivery of toxic agents or ribozymes of the invention. The invention encompasses DNA expression vectors and viral vectors that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs expression of the coding sequences and genetically engineered host cells that contain any of the foregoing coding sequences operatively
10 associated with a regulatory element that directs the expression of the coding sequences or RNAs in the host cell or pathogen. A key to the present invention is the strategy used to deliver the toxic agent and/or ribozyme to the targeted microorganism or pathogen. Two separate classes of delivery systems can be manufactured, one biologic in nature and the other abiologic.

15 Accordingly, present invention also provides the delivery of the toxic agents of the invention to cell or pathogen by abiologic or biologic systems. The present invention provides compositions of matter which has resulted from the development of methods and compositions for the delivery of one or more ribozymes and/or toxic agents directed against fundamental and essential cellular processes specific to a targeted microorganism through
20 an inactivated, altered, or modified virus (virion) or bacteriophage delivery vehicles. The present invention also provides abiologic delivery vehicles, capable of delivering a nucleic acid comprising the toxic agent(s) and/or ribozyme(s) into the targeted microorganism.

5.3.1. BIOLOGIC DELIVERY VEHICLES

25 The biologic delivery vehicle of the invention takes advantage of the fact that generalized transducing particles lack DNA originating from the viral delivery vehicle or have a reduced capacity to transfer DNA originating from the viral delivery vehicle. In a preferred embodiment of the invention, the viral delivery vehicle is a bacteriophage, or modified bacteriophage. In one embodiment, such viral or bacteriophage particles only
30 contain sequences of host origin. In other embodiments, such particles contain engineered plasmids/vectors encoding the toxic agent(s) or ribozyme(s) to be delivered. In other embodiments, such particles contain engineered plasmids/vectors encoding the toxic agent(s) or ribozyme(s) to be delivered and contain mutations which inactivate the ability of the delivery vehicle to transfer DNA originating from the delivery vehicle. Consequently,
35 the invention uses a biologic assembly of viral head proteins (packaging elements for the antimicrobial therapeutic) around the nucleic acid containing the necessary genetic elements

that will insure the desired level of expression of the toxic agent(s) and/or ribozyme(s). An important features of the present invention are the combination of toxic agents or ribozyme with viral delivery and assembly of the virions using a unique combination of plasmid features.

5 In one preferred embodiment, the invention provides bacteriophage which deliver a toxic agent of the invention. Bacteriophage of the invention may be constructed to deliver one or more toxic agents of the invention, such as one or more toxic gene products, proteins, antisense RNAs, sense RNAs, or combination thereof. In another embodiment of the invention, a host cell is constructed to express a pathogen-specific toxic agent or
10 ribozyme. In yet another embodiment of the invention, a host cell is constructed to express a repressor of a promoter used in the invention.

 In other embodiments, a host cell may be engineered to overexpress an antidote to a toxic agent such that the host cell is protected from toxicity and may be used as a producing strain, or manufacturing strain.

15 The present invention also encompasses expression systems, which may be used to express the toxic agents and/or ribozymes such as bacteriophage, viral vectors, etc. For example, a variety of bacteriophage systems may be utilized to express the selected ribozyme(s) and/or toxic agent(s) of the invention. For example, such bacteriophage systems represent vehicles by which the sequences encoding the toxic agent(s) and/or
20 ribozyme(s) may be introduced into target bacteria both *in vivo* and *in vitro*. In several embodiments, the specific bacteriophage which is selected determines the species of bacteria which is targeted and infected by that bacteriophage.

 In one embodiment, delivery of a toxic agent to a pathogen is by use of a bacteriophage or other delivery vehicle which targets the pathogen of interest. In one
25 embodiment, the bacteriophage (or delivery vehicle) delivers the toxic agent or nucleic acids encoding the toxic agent to the pathogen. In a specific embodiment, a toxic agent of the invention is delivered to a bacterial cell by a modified bacteriophage capable of infecting a pathogenic bacteria. In a further embodiment, bacteriophage are selected for their ability to infect a particular species or genera of bacteria, and are used to deliver a
30 toxic agent for the eradication of such bacterial species or genera from a host. In a preferred embodiment, the delivery vehicle or nucleic acids native to the delivery vehicle are modified such that they contain insufficient genetic information for the delivery of nucleic acids native to the delivery vehicle. Thus, the modified delivery vehicle (*e.g.*, virion or bacteriophage) can serve as a molecular vehicle that delivers the ribozyme(s) and/or toxic
35 agent(s) of the invention to the target cell or pathogen, but does not deliver replicable nucleic acids native to the delivery vehicle. Alternatively, an abiologic delivery system

(e.g., liposomes) can be used to package nucleic acid carrying the genetic elements necessary and sufficient for the proper expression of the ribozyme(s) and/or toxic agent(s).

The toxic agents and/or ribozymes of the invention may be used to treat infection from a variety of pathogens. These include but are not limited to microorganisms such as bacteria, parasites, and fungi. In specific embodiments of the invention, the toxic agents of the invention, delivered by a viral delivery vehicle (such as a modified bacteriophage) are useful to treat microbial infections associated with severe burns, cystic fibrosis, cancer, or other immunocompromising conditions.

10 **5.3.1.1. DELIVERY & EXPRESSION BY VIRAL VECTORS**

In accordance with the present invention, a wide variety of viruses and viral vectors may be used to deliver the nucleotide sequences encoding the toxic agent(s) and/or ribozymes of the present invention, a few examples of which are described below. In this regard, a variety of viruses may be genetically engineered to express the selected toxic agent(s) and/or ribozymes in order to target a specific pathogen.

The present invention also relates to the delivery of the toxic agents of the invention to cell or pathogen by abiologic or biologic systems. In a specific embodiment, For example, as described herein, a toxic agent of the invention is delivered to a bacterial cell by a bacteriophage capable of infecting a pathogenic bacteria. In a further embodiment, bacteriophage are selected for their ability to infect a particular species of bacteria, and are used to deliver a toxic agent for the eradication of such bacterial species from a host.

The invention provides for use of a virion which can also be any bacteriophage which specifically infects a bacterial pathogen of the present invention as well as any virus which can be specifically targeted to infect the pathogen of the present invention. For example, the bacteriophage can include, but is not limited to, those specific for bacterial cells of the following genera: *Bacillus*, *Campylobacter*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Mycobacterium*, *Pseudomonas*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Vibrio*, *Streptomyces*, *Yersinia* and the like (see, e.g., the American Type Culture Collection Catalogue of Bacteria and Bacteriophages, latest edition, Rockville, MD), as well as any other bacteriophages now known or later identified to specifically infect a bacterial pathogen of this invention. The invention also provides for the use of a virion which specifically infects a fungal pathogen.

This delivery system consists of a DNA plasmid carrying the nucleic acids coding for the toxic agent(s) and/or ribozyme(s) packaged into viral particles. Specificity is conferred by the promoter driving transcription of the toxic agents and/or ribozymes and by

the host specificity of the viral vehicle. Specificity is also conferred by the origin of replication controlling vector replication.

In the virions of the present invention, the non-viral DNA can encode one or more toxic agent(s) and/or one or more ribozyme(s). In the virions, the non-viral DNA can comprises a pathogen-specific or tissue-specific promoter operably linked to a sequence encoding one or more toxic agents or ribozymes.

The nucleic acid delivered by a virion can encode one or more toxic agent(s) and/or one or more ribozyme(s) or a combination thereof. The virion can comprise any nucleic acid encoding a ribozyme or toxic agent, particularly those described herein.

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Bacteriophage P1

The invention provides the use of any virion for the delivery of a toxic agent or ribozyme to a target cell. For example, a common bacteriophage of *E. coli*, P1, is an attractive delivery vehicle for the invention for a number of reasons. First and foremost, P1 has a broad intergenera and interspecies range (Yarmolinsky et al., 1988, Mol. Gen. Genet. 113:273-284). The P1 receptor of *E. coli* is the terminal glucose of the lipopolysaccharide (LPS) core lysergic ring of the bacterial outer membrane (Generalized Transduction, p. 2421-2441. In F. Neidhardt (ed.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2d ed. Vol.2, ASM Press, Washington, DC.). Yarmolinsky and Sternberg report that in addition to *E. coli*, this particular phage has the ability to inject its nucleic acid into a large number (>25) of diverse Gram negative bacteria (Yarmolinsky et al., 1988, Mol. Gen. Genet. 113:273-284). Secondly, P1 can accommodate a significant amount of genetic information, over 2% (100,000 bp) of the DNA of *E. coli* (Generalized Transduction, p. 2421-2441. In F. Neidhardt (ed.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2d ed. Vol.2, ASM Press, Washington, DC.). Consequently, gene dosage of the ribozymes or toxic agents can be increased through multiplication of the toxic agents and/or ribozymes, thereby increasing the microbicidal activity of the toxic agents and/or ribozymes. Accordingly, bacteriophage P1 is used as the delivery vehicle or molecular syringe. P1 has advantages over certain with lytic phage therapy which may harbor risk of 1) dissemination of undesirable products (e.g., DNA originating from the P1 bacteriophage) to nonpathogenic indigenous microflora, 2) excessively narrow host range, 3) rapid clearance of the material by the reticuloendothelial system of the host and 4) the concern that a lytic infection could become uncontrolled in commensal bacteria in immunocompromised patients. In certain of these embodiments, the P1 delivery system is the preferable delivery vehicle for delivery of a toxic agent to a target pathogenic bacterium. An additional advantage of the P1 delivery vehicle is that phage-mediated transfer of

undesirable products may be decreased or avoided when the phage are engineered such that they are incapable of transferring endogenous phage DNA to the host. In this embodiment, the phage particles inject transfer plasmid DNA into target bacterial cells. Expression of the encoded toxic agents may then result in bacterial cell death independent of the bacterium's resistance to antibiotics.

Additionally, a process utilizing *in vitro* packaging is also possible. *In vitro* packaging can be accomplished through the addition of PAC-sites to the genetic information of the toxic agent or ribozyme construct. P1 packaging initiates within one of the P1 PAC genes (Steinberg, N.,1987, J. Mol. Biol. 194(3) :469-79). It has been reported that the active PAC site is contained within a 161 base-pair segment of the P1 EcoRI fragment 20 (Steinberg, N.,1987, J. Mol. Biol. 194(3) :469-79). Thus, the phage head serves as a molecular syringe that delivers the inactivating ribozyme(s) and/or toxic agent(s) to the pathogen.

In specific preferred embodiments of the invention, a toxic agent is encoded in a Transfer plasmid, and is used in connection with a P1 bacteriophage delivery system. Such Transfer plasmid preferably contains 1) an origin or replication 2) selectable marker 3) Pac ABC genes with a P1 PAC site 4) P1 lytic replicon and 5) nucleic acids encoding one or more toxic agents of the invention (*e.g.*, antisense molecule, ribozyme, or toxic protein, etc). The Transfer plasmid may be produced in a bacterium producing cell (*e.g.*, a P1 lysogen). In preferred embodiments of the invention, the bacteriophage P1 plasmid (*e.g.*, the P1 prophage) is engineered to be incapable of being packaged into a phage head. In this embodiment, only Transfer plasmids are packaged into virions. Such inhibition of P1 plasmid packaging is accomplished by introducing a mutation or deletion in the P1 plasmid that inhibits the P1 plasmid from being packaged into a virion or phage head. Mutation(s) or deletion(s) of the P1 plasmid which inhibit packaging include but are not limited to one or more mutations and/or deletions in the P1 plasmid PAC site. Any mutation(s) and/or deletion(s) of the P1 plasmid which inhibits packaging of the bacteriophage P1 plasmid is within the scope of the invention. Such mutations or deletions are introduced by standard techniques known in the art. In several embodiments, the P1 lysogen has a temperature sensitive repressor mutation (*e.g.* C1.100). Preferably, induction of the P1 lysogen leads to the production of P1 phage heads containing only the packaged Transfer plasmid. Bacteriophage containing the packaged Transfer plasmid nucleic acids may then be used to infect a target cell such as a bacterial pathogen. The bacteriophage infects a bacterial pathogen by injecting its nucleic acids into the bacterium. The toxic agent encoded in the bacteriophage nucleic acids is thus delivered to the bacterium. Within the bacterium, the Transfer plasmid nucleic acids recircularize, and the toxic agent is expressed in the

5 bacterium leading to toxicity and death. Similar mutation and/or deletion strategies may be used with the other viral delivery systems of the invention such that the deletion(s) and/or mutation(s) allow packaging of the nucleic acids encoding toxic agent or ribozyme of the invention, but prevent packaging of nucleic acids encoding one or more viral genes or plasmids. Such strategies allow for construction of viral delivery systems which have increased safety (e.g., when used in connection with therapeutics of the invention).

10 In a specific embodiment, the invention provides a bacteriophage able to package/deliver Transfer plasmid in P1 virions which will infect a pathogenic bacterial target. In another specific embodiment, bacteriophage P1 (PAC site) knockouts able to package/deliver Transfer plasmid DNA but unable to incorporate P1 DNA thus preventing horizontal transfer of undesirable products to non-pathogenic indigenous microflora. In another specific embodiment of the invention, the phage delivery system comprises a Transfer plasmid carrying the genes encoding the antimicrobial agents, a plasmid origin of replication, the P1 lytic origin of replication and a minimal PAC site (e.g., such as the
15 minimal P1 pac site as shown in Figure 12, SEQ ID NO: 1). In this embodiment, the plasmid is maintained in a bacteriophage P1 lysogen unable to package its own DNA. The defective lysogen provides all the replication factors needed to activate the P1 origin of replication on the transfer plasmid and all the structural components necessary to form mature virions. The lysogen also carries the c1.100 temperature-sensitive repressor
20 mutation. C1 is responsible for the repression of functions leading to vegetative phage production. Induction of the lysogen by a temperature shift results in multiplication of DNA, packaging of the transfer plasmid into P1 phage heads and lysis of the production strain. Virions are harvested and used to deliver the Transfer plasmid to the pathogen. The phagehead contains multiple copies of Transfer plasmid DNA and is targeted to pathogenic
25 bacteria by the bacteriophage's natural receptor mediated mechanisms. Upon delivery, plasmid DNA recircularises and expression of the toxic agent under the control of environmental, virulence-regulated or species-specific promoters results in rapid cell death.

30 In specific embodiments, the invention provides novel Transfer plasmids encoding toxic agents which may be used in combination with a bacteriophage delivery system in order to treat a bacterial infection in a host.

Bacteriophage Lamda

35 Another example of a system using bacteriophage virions to package DNA carrying ribozymes and/or toxic agents directed against *E. coli* is the bacteriophage lamda. Similar strategies are used to generate virions capable of delivering ribozymes and/or toxic agents directed against other microorganisms. The virions used to package the DNA can be

species-specific, such as the virion derived from the bacteriophage lambda coat, or they can possess a broader host range, such as virion derived from bacteriophage P1, as described above. Broad host-range viruses facilitate production of the antimicrobial agents without the loss of species specificity because species-specific promoters are used to direct the transcription of the ribozymes which are directed against species-specific targeted RNA sequences. For example, a lamda bacteriophage entails the use of a plasmid carrying the ribozyme and/or toxic agent, a plasmid origin of replication, a selectable marker for plasmid maintenance, the minimal lambda origin of replication, and cos sites, which are required for packaging of DNA into lambda virions. This plasmid is maintained in a lambda lysogen that is defective in integration/excision and recombination functions. The defective lysogen provides all of the replication factors needed to activate the lambda origin of replication on the plasmid and all of the structural components needed to form mature virions; however, the lysogen is not able to replicate and package its own DNA into the virions. The lysogen may also carry a temperature-sensitive repressor mutation (such as the cI857).

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Other Viral Vectors

Retroviral vectors are also commonly used to deliver genes to host cells both *in vivo* and *ex vivo*. Retroviral vectors are extremely efficient gene delivery vehicles that cause no detectable harm as they enter the cells. The retroviral nucleic acid may integrate into host chromosomal DNA allowing for long-term persistence and stable transmission to future progeny, such a vector would be useful for the delivery of a toxic agent and/or ribozyme(s) used to target a cellular gene product involved in a chronic or hereditary disorder or to target a viral gene or a microbial gene or a parasitic gene involved in a chronic or persistent infection. An example of an appropriate retroviral vector are, lentiviruses which have the advantage of infecting and transducing non-dividing cells. In such an embodiment, a lentiviral vector encoding a packagable RNA vector genome operably linked to a promoter in which all the functional retroviral auxiliary genes are absent, is used to transfer the DNA encoding the toxic agent and/or ribozyme of the present invention. Examples of such vectors are described in WO 98/17815, WO 98/17816 and WO 98/17817, each of which is incorporated herein by reference in their entirety.

In yet another embodiment, non-integrating viral vectors which infect and transduce non-dividing cells, such as adenoviral vectors may be used to deliver the toxic agent and/or ribozymes of the present invention. Adenoviral vectors have several advantages because the use of such vectors avoids risks associated with permanently altering the host cell genome or of promoting insertional mutagenesis. Adenoviruses are one of the best developed non-integrating viral vectors and can be used to transfer expression cassettes of up to 75 kb. Recombinant adenoviruses can be produced at very

high titers, is highly infectious and efficiently transfers genes to a wide variety of non-replicating and replicating cells and is ideal for *in vivo* mammalian gene transfer.

Adenovirus-based vectors are relatively safe and can be manipulated to encode the desired toxic agent and/or ribozymes and at the same time to be inactivated in terms of their ability to replicate in a normal lytic viral life cycle. Adenovirus has a natural tropism for airway epithelia. Therefore, adenovirus-based vectors are particularly preferred for respiratory gene therapy applications. In a particular embodiment, the adenovirus-based gene therapy vector comprises an adenovirus 2 serotype genome in which the Ela and the Elb regions of the genome, which are involved in early stages of viral replication have been deleted and replaced by nucleotide sequences of interest. In a further embodiment, the adenovirus-based gene therapy vector contains only the essential open reading frame (ORF3 or ORF6 of adenoviral early region 4 (E4) and is deleted of all other E4 open reading frames, or may additionally contain deletions in the E3 regions (*e.g.*, see U.S. Patent No. 5,670,488, incorporated herein by reference in its entirety). In another embodiment, the adenovirus-based therapy vector used may be a pseudo-adenovirus (PAV), which contain no harmful viral genes and a theoretical capacity for foreign material of nearly 36 kb.

In another embodiment, adeno-associated virus (AAV) systems may be used to deliver the toxic agent and/or ribozymes of the present invention. AAV has a wide host range and AAV vectors have currently have been designed which do not require helper virus. Examples of such AAV vectors are described in WO 97/17458, incorporated herein by reference in its entirety.

Vaccinia viral vectors may be used in accordance with the present invention, as large fragments of DNA are easily cloned into its genome and recombinant attenuated vaccinia variants have been described (Meyer, et al., 1991, J. Gen. Virol. 72:1031-1038). Orthomyxoviruses, including influenza; Paramyxoviruses, including respiratory syncytial virus and Sendai virus; and Rhabdoviruses may be engineered to express mutations which result in attenuated phenotypes (see U.S. Patent Serial No. 5,578,473, issued November 26, 1996). These viral genomes may also be engineered to express foreign nucleotide sequences, such as the selected toxic agent and/or ribozymes of the present invention (see U.S. Patent Serial No. 5,166,057, issued November 24, 1992, incorporated herein by reference in its entirety). Reverse genetic techniques can be applied to manipulate negative and positive strand RNA viral genomes to introduce mutations which result in attenuated phenotypes, as demonstrated in influenza virus, Herpes Simplex virus, cytomegalovirus and Epstein-Barr virus, Sindbis virus and poliovirus (see Palese et al., 1996, Proc. Natl. Acad. Sci. USA 93:11354-11358). These techniques may also be utilized to introduce foreign DNA, *i.e.*, the selected toxic agent and/or ribozyme, to create recombinant viral vectors to

be used in accordance with the present invention. In addition, attenuated adenoviruses and retroviruses may be engineered to express the toxic agent and/or ribozymes. Therefore, a wide variety of viruses may be engineered to design the ribozymes delivery vehicles of the present invention.

5 The viral vectors of the present invention may be engineered to express the toxic agents and/or ribozymes in a tissue specific manner. For example, the promoter of the carcinoembryonic antigen (LEA) is expressed in a proportion of breast, lung and colorectal cancers, but rarely in healthy tissues. In order to target a hepatoma, the α -fetoprotein (AFP) promoter whose activity is restricted to malignant cells. Proliferating cells can be targeted
10 with a flt-1 promoter, which has been shown to allow preferential targeting of proliferating endothelial cells. See Miller et al., 1997, Human Gene Therapy 8:803-815, incorporated herein by reference in its entirety.

5.3.2. ABIOLGIC DELIVERY VEHICLES

15 Abiologic delivery of one or more toxic agents and/or ribozymes is accomplished by a variety of methods, including packaging plasmid DNA carrying the gene(s) that codes for the toxic agent(s) and/or ribozyme(s) into liposomes or by complexing the plasmid DNA carrying the gene(s) that codes for the toxic agent(s) and/or ribozyme(s) with lipids or liposomes to form DNA-lipid or DNA-liposome complexes. The
20 liposome is composed of cationic and neutral lipids commonly used to transfect cells *in vitro*. The cationic lipids complex with the plasmid DNA and form liposomes.

 A liposome is provided, comprising a nucleic acid comprising a pathogen-specific promoter operably linked to a sequence encoding a trans-acting ribozyme comprising a) a 5' autocatalytically cleaving ribozyme sequence, b) a catalytic ribozyme
25 comprising a target RNA-specific binding site and c) a 3' autocatalytically cleaving ribozyme sequence.

 A liposome is provided, comprising a nucleic acid encoding a pathogen-specific promoter operably linked to a sequence encoding one or more toxic agents is provided.

30 The liposome of the invention, wherein the nucleic acid encodes more than one trans-acting ribozyme and/or more than one toxic agent is provided. The liposome can comprise any ribozyme-encoding nucleic acid, or any toxic agent encoding nucleic agent particularly those described herein. Such nucleic acids may be operably linked to a tissue-specific or pathogen-specific promoter.

35 The liposomal delivery systems of the invention can be used to deliver a nucleic acid comprising a tissue-specific promoter operably linked to a sequence encoding a

multi-ribozyme comprising a) a 5' autocatalytically cleaving ribozyme sequence, b) a catalytic ribozyme comprising a target RNA-specific binding site and c) a 3' autocatalytically cleaving ribozyme sequence.

The liposome delivery system of the invention can be used to deliver a nucleic acid comprising a tissue-specific promoter operably linked to a sequence encoding one or more toxic agents. The liposome delivery system of the invention can be used to deliver a nucleic acid comprising a pathogen-specific promoter operably linked to a sequence encoding one or more toxic agents.

Cationic and neutral liposomes are contemplated by this invention. Cationic liposomes can be complexed with a negatively-charged biologically active molecule (*e.g.*, DNA) by mixing these components and allowing them to charge-associate. Cationic liposomes are particularly useful when the biologically active molecule is a nucleic acid because of the nucleic acids negative charge. Examples of cationic liposomes include lipofectin, lipofectamine, lipofectace and DOTAP (Hawley-Nelson et al., 1992, Focus 15(3):73-83; Felgner et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:7413; Stewart et al., 1992, Human Gene Therapy 3:267-275). Procedures for forming cationic liposomes encasing substances are standard in the art (Nicolau et al., 1987, Methods Enzymol. 149:157) and can readily be utilized herein by one of ordinary skill in the art to encase the complex of this invention.

In yet another embodiment of the present invention, the plasmid DNA carrying the gene(s) that codes for the toxic agents and/or ribozymes of the invention are complexed with liposomes using an improved method to achieve increased systemic delivery and gene expression (Templeton et al., 1997, Nature Biotechnology 15: 647-652, incorporated herein by reference in its entirety). In accordance with the present invention, an improved formulation of cationic lipids which greatly increase the efficiency of DNA delivery to host cells, with extended half-life *in vivo* and procedures to target specific tissues *in vivo*. For example, but not by limitation, peptides and proteins may be engineered to the outer lipid bilayer, such as liver-specific proteins, leads to substantially enhanced delivery to the liver etc.

In one embodiment of the present invention, systemic delivery and *in vivo* and *ex vivo* gene expression is optimized using commercially available cationic lipids, *e.g.*, dimethyldioctadecylammonium bromide (DDAB); a biodegradable lipid 1, 2-bis(oleoyloxy)-3-(trimethylammonio) propane (DOTAP); these liposomes may be mixed with a neutral lipid, *e.g.*, L- α dioleoyl phosphatidylethanolamine (DOPE) or cholesterol (Chol), two commonly used neutral lipids for systemic delivery. DNA:liposome ratios may be

optimized using the methods used by those of skill in the art (e.g., see Templeton et al., 1997, Nature Biotechnology 15: 647-152, incorporated herein by reference in its entirety).

In yet another embodiment of the present invention, the plasmid DNA carrying the nucleic acids encoding the toxic agents and/or ribozymes of the invention may
5 be delivered via polycations, molecules which carry multiple positive charges and are used to achieve gene transfer *in vivo* and *ex vivo*. Polycations, such as polyethylenimine, may be used to achieve successful gene transfer *in vivo* and *ex vivo* (e.g., see Boletta et al., 1996, J. Am. Soc. Nephrol. 7: 1728, incorporated herein by reference in this entirety.)

The liposomes may be incorporated into a topical ointment for application or
10 delivered in other forms, such as a solution which can be injected into an abscess or delivered systemically, or delivered by an aerosol.

Plasmid DNA coding for the ribozymes or toxic agent is used rather than preformed ribozymes or toxic agent for the following reasons. Plasmid DNA allows the targeted cells to produce the toxic agent or ribozyme and, thus, results in a higher delivered
15 dose to the cell than can be expected by delivery of ribozyme RNA or toxic agent via liposome. The DNA also provides specificity of action based on target sequence specificity. The liposomes deliver their DNA to any cell in the area of administration, including cells of the host. The promoter driving the transcription of the toxic agent or ribozyme is specific for the targeted microorganism and, thus, will be inactive in other cell types. Therefore,
20 liposomal delivery of DNA coding for the toxic agent or ribozyme provides amplification and specificity. The present invention relates to promoter elements which are pathogen-specific or tissue-specific. Such promoter elements are used to achieve pathogen-specific or tissue-specific expression of the toxic agent(s) and/or ribozyme(s) of the present invention. The invention also relates use of an origin of replication which modulates specificity of the
25 replication or copy number of a vector or plasmid in a cell or pathogen.

5.3.2.1. DELIVERY & EXPRESSION USING MULTI-RIBOZYMES

In another embodiment of the invention, expression of a toxic agent is directed by a tissue-specific, pathogen-specific, and/or target-specific ribozyme or ribozyme
30 cassette. The invention provides ribozymes that have the unique characteristic of being both target RNA-specific in their catalytic action, and tissue-specific or pathogen-specific in their expression. A ribozyme can be tissue-specific in the case of treating tissue-specific disease, or it can be pathogen-specific in the case of treating a pathogen such as *E. coli*. Multi-ribozymes may have one or more target-specific ribozyme(s) (e.g., a trans-acting
35 catalytic ribozyme) as well as elements which control tissue-specific or pathogen-specific expression.

In one embodiment, the nucleic acids of the invention comprise a tissue-specific promoter operably linked to a sequence encoding a) a 5' autocatalytically cleaving ribozyme sequence, b) a catalytic ribozyme comprising a target RNA-specific binding site and c) a 3' autocatalytically cleaving ribozyme sequence.

5 The tissue-specific promoter in the ribozyme-producing construct results in tissue-specific expression of the ribozyme in tissue(s) that actively transcribe RNA from the selected promoter. Thus, only the target RNA in tissue that utilize the promoter will be cleaved by the ribozyme.

Further, in accordance with the present invention, the multi-ribozyme may
10 consist of one or more ribozyme cassettes. Each cassette in turn may consist of a catalytic core and one or more flanking sequences. In one embodiment, the ribozyme cassette may consist of a 5' autocatalytically cleaving ribozyme sequence, a core catalytic ribozyme comprising a trans-acting ribozyme and a 3' autocatalytically cleaving ribozyme. In yet another embodiment, the catalytic core contains sequences encoding one or more toxic
15 agent(s). In other embodiments, the multi-ribozymes comprise a cassette including, the enhanced 5' and 3' autocatalytically cleaving ribozyme sequence. In another embodiment, the multi-ribozymes contain one or more internal trans-acting ribozymes. In a preferred embodiment, the multi-ribozymes of the present invention include, but are not limited to triple ribozyme cassettes. In another embodiment, multi-ribozymes include but are not
20 limited to one or more triple ribozyme cassettes linked together. In yet another embodiment, the multi-ribozyme comprises a ribozyme cassette containing one or more internal trans-acting ribozyme. In an additional embodiment, the multi-ribozyme comprises a series of one or more ribozyme cassettes containing one or more internal trans-acting ribozymes or any combination thereof. In further embodiments, the multi-ribozyme
25 cassettes or toxic agent(s) are expressed in a tissue-specific or pathogen-specific manner. In a preferred embodiment of the invention, pathogen-specific expression is coupled to a pathogen-specific promoter.

5.4. PROMOTER SELECTION

30 Promoter selection is accomplished using techniques that are available in the art. As used herein, regulatory elements include but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Specifically, the invention provides inducible promoters which have increased transcriptional control and high expression levels.
35 The promoter can be a naturally occurring strong, intermediate or weak constitutively expressed or regulated promoter from the targeted microorganism, or an artificially

contrived constitutively expressed or regulated promoter containing either a strong, intermediate or weak consensus sequence that delivers desired levels of toxic agent or ribozyme expression in the targeted microbe.

- Promoters specific for the target (*e.g.*, a specific pathogen, genus, etc.) in question can be selected by screening genomic sequences for the ability to activate a promoterless reporter gene. The promoterless reporter gene is based on the strategy developed for use with plasmid pMC1871 (Casadaban et al., 1983, *Meth. Enzymol.* 100:293). For non-viral pathogens, plasmid capable of stable replication and maintenance in the microorganism under study is modified by standard molecular biology techniques to carry the coding region of a reporter gene (Sambrook et al. latest edition). The reporter gene can be any of a number of standard reporter genes including but not limited to the *lacZ* gene of *E. coli*, which codes for β -galactosidase. Total genomic DNA is isolated from cells of the pathogen, cleaved with restriction endonucleases to yield fragments of a few hundred basepairs on average. These fragments are then ligated into a unique restriction endonuclease cleavage site at the 5' end of the reporter gene coding region, creating a library of plasmids. The library is then transformed into the pathogen by standard techniques and the resulting transformants are screened for expression of the reporter gene. In the case of *lacZ*, the transformants can be plated onto medium containing the chromogenic galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside). Transformants that contain a plasmid with an insert carrying a promoter will express β -galactosidase and will turn blue on X-Gal plates. The intensity of the blue color is relative to the level of expression; promoters of different strength can be selected based on the intensity of the blue color.

- The above-described screening procedure can be modified to identify regulated promoters. For example, promoters that are regulated by carbon source availability can be screened on plates that contain different carbon sources. Other modifications are possible and will depend, in part, on the organism in question. To test for species-specificity, the identified promoters are transferred to promoterless reporter plasmids capable of replication and maintenance in a different organism. Truly species-specific or pathogen-specific promoters will not activate the expression of the reporter gene in any other species. Obvious modifications can be used to identify and test artificial promoters composed of synthetic oligonucleotides inserted into the promoterless reporter plasmid.

- In one embodiment, the nucleic acids of the invention comprise a tissue-specific promoter operably linked to a sequence encoding a 5' autocatalytically cleaving

ribozyme sequence, one or more catalytic target-specific trans-acting ribozymes or one or more toxic agents and a 3' autocatalytically cleaving ribozyme sequence.

The tissue-specific promoter in the ribozyme-producing construct results in tissue-specific expression of the ribozyme in tissue(s) that actively transcribe RNA from the selected promoter. Thus, only the target RNA in tissue that utilize the promoter will be cleaved by the ribozyme. The pathogen-specific promoter binding site in the ribozyme-producing construct results in pathogen-specific expression of the ribozyme in pathogens or microbes that actively transcribe RNA from the selected promoter. Thus, only the target RNA in pathogens that utilize the promoter will be cleaved by the ribozyme.

Tissue-specific promoters can be used in the present nucleic acid constructs.

Examples of these promoters include the sequences for probasin-promoter, a promoter-specific for prostate epithelium prostate-specific antigen (prostate), keratin, k7 (epidermal sabaceus glands), albumin (liver), fatty acid binding protein (ileum), whey acidic protein (breast), lactalbumin, smooth muscle actin (smooth muscle), etc. In a specific embodiment,

a mouse albumin promoter/enhancer is used which consists of nucleotides 338-668 from Genbank accession # U04199, followed by the sequence gagtcgacggatccgg, followed by nucleotides 1762-1846 from accession # J04738, followed by the nucleotide sequence tgggggtgggggtggggg, followed by nucleotides 1864-2063 of accession # J04738. In one embodiment, the mouse promoter/enhancer is active in hepatocyte (e.g., human hepatocytes, hepatocyte cultures, etc.) and is useful for tissue-specific expression in liver tissue. It will also be clear that target-specific promoters not yet identified can be used to target expression of the present ribozymes to the selected tissue(s). Once a target-specific promoter is identified its binding sequence can be determined by routine methods such as sequence analysis may be used. The promoter is defined by deletion analysis, mutagenesis, footprinting, gel shifts and transfection analyses (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). Pathogen-specific promoters can be used in the present nucleic acid constructs.

5.4.1. BACTERIAL SPECIFIC PROMOTERS/EXPRESSION

The present invention provides bacterial promoters that allow for tight regulation of transcription and enhanced expression. In one embodiment, a novel promoter called LEASHI has been constructed from three elements (see Figure 1). The first element, termed RIP is a combination of two consensus sites at -10(TATAAT) and -35(TTGACA) located with respect to transcription initiation. The second element is based on the lacI repressor binding sequence (termed lac operator sequence) which is placed

between the -10 and -35 consensus sites. This is in contrast to the conventional lac and tac promoters where the lac operator is found downstream of the -10 consensus element. Placement of the lac operator between the -10 and -35 sites, more effectively blocks RNA polymerase binding to the promoter, thus enhancing transcriptional control from the promoter. Thus, the levels of lacI repressor protein present, which binds to the operator sequence and hence determines the rate of transcription, are controlled in two ways; 1) by endogenously expressed lacI protein and 2) by a plasmid expressing the lacI gene. Under normal conditions, the lacI repressor protein binds to the lac operator sequence and prevents transcription by blocking RNA polymerase binding. The promoter is 'switched on' following the addition of isopropyl B-D-thiogalacto pyranoside, which binds and subsequently titrates out the repressor protein. RNA polymerase can then bind to the promoter and transcription can proceed.

The third element of the LEASHI promoter is termed the UP element. The UP element is an adenine/thymine rich sequence which is placed immediately upstream of the -35 element. Addition of the UP element, further increases expression from this promoter. Accordingly, the invention provides the use of a LEASHI promoter to express the toxic agents of the invention.

In a specific embodiment of the invention, the promoter which is operably linked to a nucleic acid encoding a toxic agent or ribozyme is the LEASHI promoter. In specific embodiment, a ribozymes of the invention is operably linked to a LEASHI promoter. In another specific embodiment of the invention, a toxic agent of the invention is operably linked to a LEASHI promoter.

In a specific embodiment, the invention encompasses expression of *DicF1* from a ribozyme cassette under the control of a regulatable promoter, such as the LEASHI promoter.

In another embodiment of the invention, the lacI operator sequence of the LEASHI promoter is placed 5' of the -35 consensus site. In another embodiment of the invention, the lacI operator sequence of the LEASHI promoter is placed 3' of the -10 consensus site. In other embodiment of the invention, one or more additional lacI operator sequences are added to the LEASHI promoter and are placed 5' to the -35 consensus site and/or 3' of the of the -10 consensus site.

In other specific embodiments, the invention provides for the use of an *anr*, *arc*, or *proC* promoter. Both are transcriptionally off in *E. coli* and on in *Pseudomonas aeruginosa*. These promoters provide the advantage of allowing controlled expression of the toxic agents in the pathogen (*Pseudomonas*), while allowing the packaging strain (*E. coli*) to be protected from the toxic actions of the toxic agent therapeutic. Such promoters

are particularly useful to facilitate manufacturing of the delivery vehicle. Such promoters also enable bacterial specific targeting of the gene therapeutic in the patient. In specific embodiments, an anr promoter is operably linked to a sequence encoding a toxic agent (such as *doc*, *gef*, *chpBK*, or *kicB*, etc), and may be used, for example, for the eradication of

5 *Pseudomonas*.

In other specific embodiments, the invention provides for the use of the *TSST-1* promoter. *TSST-1* is an environmentally regulated staphylococcus-specific promoter. *TSST-1* is useful to express *doc* or other toxic agents. A staphylococcus specific phage capable of delivering the transfer plasmid into *S. aureus* strains is used to specifically

10 target the Staphylococcal pathogen.

Other classical bacterial inducible promoters are renowned for their inability to tightly control transcription, and a significant level of background expression is characteristically observed. A significant advantage of the promoter of the present invention is that it will alleviate the high levels of background commonly observed in

15 inducible promoters. A limiting factor leading to high background levels of transcription when a promoter of interest is on a high-copy number plasmid is due to the lack of repressor molecules available to bind to the promoters. The present invention overcomes this problem by using a *lacI* expression plasmid and secondly, by placement of the *lac* operator between the -35 and -10 consensus elements which more effectively blocks transcription

20 during normal conditions. Furthermore, the UP element placed immediately upstream of the -35 region enhances transcription from the core promoter.

The invention also relates to the *rrnB* promoter. In one embodiment of the invention, the promoter is the *rrnB* promoter is modified such that one or more *lacI* operator sites are added to the promoter. An example of such a modified *rrnB* promoter is shown in

25 Figure 1B. In another embodiment of the invention, the *lacI* operator sequence of the *rrnB* promoter is placed 3' of the -10 consensus site. In other embodiment of the invention, one or more additional *lacI* operator sequences are added to the *rrnB* promoter and are placed 5' to the -35 consensus site and/or 3' of the of the -10 consensus site.

30 5.5. HOST CELLS

The present invention encompasses the expression of the toxic agents and/or ribozymes in primary cells, animal, insect, fungal, bacterial, and yeast cells for *in vitro* screening assay and *ex vivo* gene therapy. The present invention also encompasses the expression of the toxic agents and/or ribozymes in cell lines for *in vitro* screening assay and

35 *ex vivo* gene therapy. In accordance with the present invention, a variety of primary or secondary cells or cell strains may be used including but not limited to cells isolated from

skin, bone marrow, liver, pancreas, kidney, adrenal and neurological tissue to name a few. Other cell types that may be used in accordance with the present invention are immune cells (such as T-cells, B-cells, natural killer cells, etc.), macrophages/monocytes, adipocytes, pericytes, fibroblasts, neuronal cells, reticular cells etc. In a further embodiment, secondary cell lines may be used as engineered responsive cells and tissues in accordance with the present invention, including, but not limited to hepatic cell lines, such as CWSV, NR, Chang liver cells, or other cell lines such as CHO, VERO, BHK, Hela, COS, MDCK, 293, 373, HUVEC, CaSki and W138 cell lines. A toxic agent or ribozyme of the invention may also be expressed in any cell line which is not sensitive to the effects of the toxic agent or ribozyme (*e.g.*, a cell which is resistant to the particular toxic agent or ribozyme, or a cell which co-expresses a neutralizing agent or antidote).

For long term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the selected toxic agent and/or ribozyme may be engineered. When a toxic agent is to be stably expressed, expression may be controlled by an inducible promoter, or, the cell may be engineered to co-express an antidote to the toxic agent, in order to allow the cell to survive during production of a toxic agent. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter sequences, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines. This method may advantageously be used to engineer cell lines which express the selected gene products. Such cell lines would be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the selected gene product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in *tk*⁻, *hgp*^{rt} or *ap*^{rt} cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: *dhfr*, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); *gpt*, which confers resistance to

mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

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5.6. TARGETS

The present invention provides a toxic agent or a trans-acting ribozyme which targets any cellular, viral, bacterial, fungal, or other single cellular or multicellular organism from any known taxonomic family, genus, or species. Another embodiment of the invention provides a toxic agent which is lethal or toxic to a pathogen such as a bacteria, fungus, yeast, diseased cell. Such toxic agents may be delivered to the pathogen by the methods of the invention. The microorganisms may be any virus, nonvirus, bacterium, or lower eukaryotes such as fungi, yeast, parasites, protozoa, or other eukaryotes that may be considered pathogens of humans, animals, fish, plants, or other forms of life. In several
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embodiments, the targets of the antimicrobial ribozyme therapeutics described herein are the RNAs of invading or normal flora microorganisms. In other embodiments, the targets of the antimicrobial toxic agent therapeutics described herein include RNAs, proteins, genes and other molecules of invading or normal flora microorganisms. Thus, the invention has important implications in human and veterinary medicine.

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The toxic agents of the present invention may be engineered to target essential genes, gene products, or processes necessary for growth of parasites, bacteria, virus life cycles, etc., and expression can be driven with tissue-specific or pathogen-specific promoters. The toxic agents or trans-acting catalytic ribozymes of the present invention may be engineered to target a wide variety of cellular RNAs, tumor or cancer associated
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with RNAs, bacterial RNAs, parasitic RNA etc. For example, ribozyme targets sites are indicated in Tables 11, 13 and 13 herein. The toxic agent or trans-acting ribozyme can be targeted to noncellular RNAs necessary for growth of parasites, bacteria, virus life cycles, etc., and expression can be driven with tissue-specific or pathogen-specific promoters.

The virion construct used in this method can comprise any nucleic acid
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encoding a toxic agent or ribozyme, particularly those described herein targeted to essential genes of the pathogen or diseased cell. The virion can be a bacteriophage, or other virus selected for its ability to target a specific cell-type, microorganism or animal. The bacteriophage can be lambda, P1, Phi-11 or other phage. When P1 is the virion, the Transfer plasmid can further comprise a PAC site and PAC ACB genes. This construct is preferred
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when using P1. Alternatively, the virion can be selected because it has a broad range of targets.

Important examples which are specifically presented in the application are:

- A) Use of the LEASHI promoter with a Bacterial target (such as *E. coli*) to direct expression of the toxic agent such as *doc*, *gef*, *chpBK*, *kicB* or *DicF1*;
- B) Use of the LEASHI promoter with a Bacterial target (such as *E. coli*) to direct expression of the toxic agent comprising *Sof* sense RNA;
- C) Use of the *anr*, *arc*, or *proC* promoter with a *Pseudomonas* target (such as *P.aeruginosa*) to direct expression of a toxic agent such as *doc*, *gef*, *chpBK*, *kicB* or *DicF1*;
- D) Use of the *TSST-1*, *hla*, or *SrcB* promoter with a *Staphylococcus* target (such as *S. aureus*) to direct expression of the toxic agent such as *doc*, *gef*, *chpBK*, *kicB*, *pemK*, *hok*, *relF*, *sigB*, or *lysostaphin*;
- E) Use of the albumin promoter with a Hepatitis B virus target (chosen to cleave the viral RNA pregenome, S protein, and polymerase/and x protein transcripts using the same ribozyme target site);
- F) Use of the albumin promoter with Hepatitis B virus and Hepatitis C targets (using trans-acting ribozyme target sites on both Hepatitis B virus and Hepatitis C);
- G) Use of generic promoters active in erythrocytes, using a ribozyme targeted to highly conserved regions of the EMP-1 protein family from *P. falciparum*, which are necessary for cytoadherence and antigenic variation in malaria; and
- H) Use of the keratin 7 promoter, with trans-acting ribozymes targeted to a specific sites near the translational start site of the E6 protein, a site known to be critical for expression of both the E6 and E7 proteins which are intimately involved in cervical carcinogenesis, as well as a more 3' site in a highly conserved region of the E6 protein.

Examples of bacterial pathogens that can be targeted by a toxic agent or trans-acting ribozyme of the present invention include, but are not limited to, species of the following genera: *Salmonella*, *Shigella*, *Chlamydia*, *Helicobacter*, *Yersinia*, *Bordatella*, *Pseudomonas*, *Neisseria*, *Vibrio*, *Haemophilus*, *Mycoplasma*, *Streptomyces*, *Treponema*, *Coxiella*, *Ehrlichia*, *Brucella*, *Streptobacillus*, *Fusospirocheta*, *Spirillum*, *Ureaplasma*, *Spirochaeta*, *Mycoplasma*, *Actinomycetes*, *Borrelia*, *Bacteroides*, *Trichomonas*, *Branhamella*, *Pasteurella*, *Clostridium*, *Corynebacterium*, *Listeria*, *Bacillus*, *Erysipelothrix*, *Rhodococcus*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Enterobacter*, *Serratia*, *Staphylococcus*, *Streptococcus*, *Legionella*, *Mycobacterium*, *Proteus*,

Campylobacter, *Enterococcus*, *Acinetobacter*, *Morganella*, *Moraxella*, *Citrobacter*, *Rickettsia*, *Rochlimea*, as well as bacterial species such as: *P. aeruginosa*; *E. coli*, *P. cepacia*, *S. epidermis*, *E. faecalis*, *S. pneumonias*, *S. xylosus*, *S. aureus*, *N. meningitidis*, *S. pyogenes*, *Pasteurella multocida*, *Treponema pallidum*, and *P. mirabilis*.

5 The pathogen of the present invention can also include, but is not limited to pathogenic fungi such as *Cryptococcus neoformans*; *Blastomyces dermatitidis*; *Aiellomyces dermatitidis*; *Histoplasma capsulatum*; *Coccidioides immitis*; *Candida species*, including *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii* and *C. krusei*, *Aspergillus species*, including *A. fumigatus*, *A. flavus* and *A. niger*, *Rhizopus species*; *Rhizomucor*
10 *species*; *Cunninghammella species*; *Apophysomyces species*, including *A. saksenaea*, *A. mucor* and *A. absidia*; *Sporothrix schenckii*, *Paracoccidioides brasiliensis*; *Pseudallescheria boydii*, *Torulopsis glabrata*; *Trichophyton species*, *Microsporum species* and *Dermatophytes species*, as well as any other yeast or fungus now known or later identified to be pathogenic.

15 Furthermore, the pathogen of the present invention can be a parasite, including, but not limited to, members of the Apicomplexa phylum such as, for example, *Babesia*, *Toxoplasma*, *Plasmodium*, *Eimeria*, *Isospora*, *Atoxoplasma*, *Cystoisospora*, *Hammondia*, *Besnotia*, *Sarcocystis*, *Frenkelia*, *Haemoproteus*, *Leucocytozoon*, *Theileria*, *Perkinsus* and *Gregarina spp.*; *Pneumocystis carinii*; members of the Microspora phylum
20 such as, for example, *Nosema*, *Enterocytozoon*, *Encephalitozoon*, *Septata*, *Mrazekia*, *Amblyospora*, *Ameson*, *Glugea*, *Pleistophora* and *Microsporidium spp.*; and members of the Ascetospora phylum such as, for example, *Haplosporidium spp.*, as well as species including *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malaria*; *Toxoplasma gondii*; *Leishmania mexicana*, *L. tropica*, *L. major*, *L. aethiopica*, *L. donovani*, *Trypanosoma cruzi*,
25 *T. brucei*, *Schistosoma mansoni*, *S. haematobium*, *S. japonium*; *Trichinella spiralis*; *Wuchereria bancrofti*; *Brugia malayi*; *Entamoeba histolytica*; *Enterobius vermicularis*; *Taenia solium*, *T. saginata*, *Trichomonas vaginatis*, *T. hominis*, *T. tenax*; *Giardia lamblia*; *Cryptosporidium parvum*; *Pneumocystis carinii*, *Babesia bovis*, *B. divergens*, *B. microti*, *Isospora belli*, *L. hominis*; *Dientamoeba fragilis*; *Onchocerca volvulus*; *Ascaris*
30 *lumbricoides*; *Necator americanis*; *Ancylostoma duodenale*; *Strongyloides stercoralis*; *Capillaria philippinensis*; *Angiostrongylus cantonensis*; *Hymenolepis nana*; *Diphyllobothrium latum*; *Echinococcus granulosus*, *E. multilocularis*; *Paragonimus westermani*, *P. caliensis*; *Chlonorchis sinensis*; *Opisthorchis felinae*, *G. Viverini*, *Fasciola hepatica*, *Sarcoptes scabiei*, *Pediculus humanus*; *Phthirus pubis*; and *Dermatobia hominis*,
35 as well as any other parasite now known or later identified to be pathogenic.

Examples of viral pathogens include, but are not limited to, retroviruses (human immunodeficiency viruses), herpes viruses (herpes simplex virus; Epstein Barr virus; varicella zoster virus), orthomyxoviruses (influenza), paramyxoviruses (measles virus; mumps virus; respiratory syncytial virus), picorna viruses (Coxsackie viruses; rhinoviruses), hepatitis viruses (hepatitis C), bunyaviruses (hantavirus; Rift Valley fever virus), arenaviruses (Lassa fever virus), flaviviruses (dengue fever virus; yellow fever virus; chikungunya virus), adenoviruses, birnaviruses, phleboviruses, caliciviruses, hepadnaviruses, orbiviruses, papovaviruses, poxviruses, reoviruses, rotaviruses, rhabdoviruses, parvoviruses, alphaviruses, pestiviruses, rubiviruses, filiviruses, coronaviruses, as well as any virus of the family of picornaviridae; caliciviridae; togaviridae; flaviviridae; coronaviridae; rhabdoviridae; filoviridae; paramyxoviridae; orthomyxoviridae; bunyaviridae; arenaviridae; reoviridae; retroviridae; hepadnaviridae; parvoviridae; papovaviridae; adenoviridae; herpesviridae; and poxyviridae, and any other virus now known or later identified to be pathogenic.

5.7. TARGET SELECTION

One critical component in the development of the therapeutics of the invention is the selection of appropriate targets.

20 *Toxic Agent Targets*

The inventions toxic agents are selected based on their ability to inhibit the growth of a pathogen or selected cell or cause lethality in a pathogen or selected cell, or render a pathogen or selected cell less fit. Several specific examples of toxic agents are described herein which serve to illustrate the selection of a toxic agent of the invention.

For example, a toxic agent may be an addiction system toxin (such as *doc*). *Doc* encodes a toxin which is translationally coupled to a protein called *phd*. *Phd* is an antidote to *doc*, and acts to neutralize the toxic effects of *doc*. The two proteins, *phd* and *doc* form an operon on the P1 plasmid in which *phd* precedes *doc*. Further, the *phd* gene contains a ribosome entry site and is translated efficiently. The native *doc* gene however, lacks a recognizable ribosome entry site and is translated poorly. Thus, *doc* was selected because of its potential toxicity when expressed in a cell or pathogen lacking the corresponding antidote, *phd*. In this embodiment, *doc* has been engineered to be uncoupled from *phd*. For example, *doc* is engineered into a separate plasmid from *phd*. The plasmid containing *doc* has also been engineered such that a ribosome entry site has been constructed upstream of the nucleic acids encoding *doc* in order to increase the levels of translation of *doc*. This plasmid is containing the toxic agent and/or ribozyme of the

invention is called the Transfer plasmid. In one specific embodiment of the invention, the Transfer plasmid encodes the toxic agent *doc*.

5 A packaging strain (*e.g.*, bacteria cell) is then used to package the Transfer plasmid containing *doc* into a bacteriophage phage head. The packaging strain cells contain the P1 plasmid as well as the Transfer plasmid with the uncoupled *doc* and ribosome entry site. The packaging strain may also include a third plasmid, if necessary, which encodes additional *phd* protein which can act to protect the packaging strain against the toxicity of *doc* (*e.g.*, if the promoter of the Transfer plasmid is leaky and leads to production of *doc* in the packaging cell).

10 Thus, the packaging strain acts to package the transfer plasmid containing the toxic agent (such as *doc*) into phage heads or virions. Phage lysates of the packaging strain contain the infectious bacteriophage virions.

The phage lysates are then used to infect a selected pathogen (*e.g.*, *E. coli*, *P. aeruginosa*, *etc.*). Further, the phage lysate may be used to prepare a therapeutic of the invention, such as a pharmaceutical preparation. Phage may be delivered to a bacteria or pathogen or a host with a pathogenic infection by methods described herein, or by any method known in the art. For example, the phage lysates may be lyophilized and delivered to a host in need of treatment for a bacterial infection, fungal infection, *etc.*

20 The above targeting method, wherein the virion is a bacteriophage is provided. The bacteriophage can be lambda, P1 or other phage. The targeting method, wherein the Transfer plasmid further comprises a PAC site and PAC ABC genes is also provided. The bacteriophage P1 which is engineered to be packaging deficient is also provided. This construct is preferred when using P1.

25 ***Antisense Targets***

A toxic agent of the invention may be an antisense molecule selected to target an antidote of a toxic protein, or selected to target an essential RNA critical to the survival of a pathogen or selected cell. The proposed target of the toxic antisense molecule of the invention may also be the RNA of any gene which plays a critical role in the survival of the pathogen, or which is essential to the pathogen's life cycle. The present invention also encompasses modifications to naturally occurring antisense molecules which modulate the expression of an essential gene product of a pathogen. For example, as described below, one proposed target of an antisense of the invention is the *ftsZ* gene whose gene product plays a critical role in the initiation of cell division of *E. coli*. For example, the toxic agent may be an antisense molecule which is constructed to be modified and enhanced such that it is more homologous to its target RNA. Thus, as in the case of *DicF*, the antisense sequence

has been modified and enhanced to engineer the *DicF1* antisense toxic agent, which has increased complementarity to its target RNA. Further, the *DicF1* or *DicF1*-like antisense molecule has enhanced properties in that it may be expressed and delivered by the methods of the invention, thus providing the target cell with increased levels of the toxic antisense RNA.

Third, a toxic agent may be selected to target an essential antisense molecule. Thus, a toxic agent may be a sense molecule which is designed to be complementary to an essential antisense RNA. An example of an essential antisense molecule is *Sof*. *Sof* is an antisense antidote for the chromosomally encoded toxin called *gef* (Poulsen, L., et al., 1991, Mol. Microbiology 5:1639-48). *Sof* normally acts to regulate the levels of *gef* in the bacterium. The inventors of the present invention have designed sense molecules which are complementary to *Sof*. The sense molecules against *Sof* act to inhibit the ability of *Sof* to regulate *gef*, and thus cause toxicity in the pathogen by allowing the endogenous *gef* levels to become toxic.

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Ribozyme Targets

For ribozymes to be effective anti-microbial therapy, it is preferable to target the RNA of, for example, several key proteins, tRNA, rRNA or any other RNA molecule essential for cell viability or fitness, in order to insure complete inactivation and prevent escape of the invading microorganism.

The complexity of human RNA is about 100 fold lower than that for human DNA, and specificity can be achieved with as few as 12-15 base pairs. The stability of the RNA-RNA duplex is effected by several factors, such as GC content, temperature, pH, ionic concentration, and structure. The nearest neighbor rules can provide a useful estimate of the stability of the duplex (Castanotto et al. "Antisense Catalytic RNAs as Therapeutic Agents" *Advances in Pharmacol.* 25:289-317, 1994).

The catalytic ribozyme of the invention also includes a catalytic sequence, which cleaves the target RNA near the middle of the site to which the target RNA-specific sequences bind. In the hammerhead-type of ribozyme, the catalytic sequence is generally highly conserved. The conserved catalytic core residues are 5' CUGANGA 3' and 5' GAAA 3' linked by an evolutionarily conserved stem-loop structure.

The most conserved and probably most efficiently cleaved sequence on the target RNA is 5' GUC 3'. However, NUX (wherein X = A, U or C) can also be cleaved efficiently. Such cleavage sites are ubiquitous in most RNAs allowing essentially all RNA's to be targeted (Whitton, J. Lindsay "Antisense Treatment of Viral Infection" *Adv. in Virus Res.* Vol. 44, 1994).

With regard to the selection of the appropriate sites on target RNA, it is known that target site secondary structure can have an effect on cleavage *in vitro* (Whitton, 1994 supra). A number of procedures are available to select accessible sites in RNA targets. In a preferred procedure, a library screen may be employed to select appropriate sites on the target RNA. Accessibility of the selected site may then be confirmed using techniques known to those skilled in the art. Thus, the selected target molecule's sequence can be routinely screened for potential secondary structure, using the program RNAFOLD (from the PCGENE group of programs or available on the Internet). Thus, reasonable predictions of target accessibility can be made. Computer assisted RNA folding (Castanotto et al., 1994), along with computational analysis for 3-dimensional modeling of RNA (Major et al., *Science* 253:1255-1260, 1991 and Castanotto et al., 1994) is certainly effective in guiding the choice of cleavage sites.

The nucleic acid, wherein at least one trans-acting ribozyme is targeted to a *ccdA*, *kis*, *pemI*, *parD*, *phd*, *higA*, *chpAI*, *chpBI*, *kicA*, *soc*, *sos*, *srnC*, *flmB*, *pndB*, *sof*, *korA*, *korB*, *korC*, *korD*, *korE*, or *korF* transcript of the pathogen is provided. The nucleic acid, wherein at least one trans-acting ribozyme is targeted to the *rpoA* transcript of the pathogen is provided. The nucleic acid, wherein at least one trans-acting ribozyme is targeted to the *secA* transcript of the pathogen is provided. The nucleic acid, wherein at least one trans-acting ribozyme is directed to the *dnaG* transcript of the pathogen is provided. The nucleic acid, wherein at least one trans-acting ribozyme is directed to the *ftsZ* transcript of the pathogen is provided. A nucleic acid encoding a multi-ribozyme can encode all or some of the above trans-acting ribozymes. The ribozymes can all be under the control of a single promoter.

For example, several bacterial genes, essential for viability and unrelated in activity, have been selected and are described herein to highlight how the selection of appropriate mRNA targets is carried out for the preferred construction of the antimicrobial agent against prokaryotic targets. Cross-genera RNA targets can be used to design an antimicrobial that can have broad application, modified by the specificity of the promoter. In addition, several toxic agents are described herein to highlight how the selection of appropriate toxic agents is carried out for the preferred construction of the antimicrobial agent against prokaryotic targets.

In one embodiment of the invention, the first ribozyme targets an essential transcription factor, the second ribozyme targets an essential general secretory component, the third ribozyme targets an essential component of the primosome required for DNA biosynthesis and the fourth ribozyme targets an enzyme required for cell division. Consequently, the ribozymes are redundant in the fact that they inhibit growth by

specifically targeting a fundamental process required for bacterial growth. Thus, this can minimize the development of resistance to the antimicrobial therapeutic.

For example, one target is the essential protein, *rpoA* or the alpha subunit of RNA core polymerase. *rpoA* was selected rather than the other components of the RNA polymerase holoenzyme, because it is thought to facilitate the assembly of an active RNA polymerase enzyme complex. Inactivation of the *rpoA* transcript results in a decrease in the intracellular concentration of the holoenzyme RNA polymerase rendering the cell less able to respond to changes demanded of it once it has invaded a new host. The nucleotide sequence of *rpoA* is known for a large number of microorganisms (>20 genera) and they are readily available from GenBank.

A second example of a ribozyme target can be the mRNA of the *secA* gene from bacteria. The product of this gene is the essential and rate-limiting component of the general secretory pathway in bacteria (Bassford et al., 1994, Nucleic Acids Research Apr. 11, 22(7):1326; Nucleic Acid Research. 22(3):293-300). *SecA* has been found in every prokaryotic cell investigated to date. Additionally, its biosynthesis is translationally coupled to the upstream gene, X (Schmidt et al., 1991, J. Bacteriol. 173(20):6605-11), presenting a convenient target for a ribozyme. Inhibition or decreased synthesis of *secA* is also sufficient to confer a reduction in viability to the cell (Schmidt et al., 1987, J. Bacteriol. 171(2):643-9). Furthermore, as a pathogen responds to changes required of the infectious process a change in the availability of a key protein such as *secA* will disadvantage the pathogen enabling the host to counteract it. Finally, control over the secretion-responsive expression of *secA* is at the level of translation (Christoffersen et al., 1995, J. Med. Chem. 38(12):2023-37), and the regulatory sequences within its polycistronic message have been localized to a region comprised of the end of the upstream gene, X, and the beginning of *secA*. Consequently, inactivation of the transcript by the catalytic cleavage of a ribozyme has profound consequences for the viability of the invading microorganism.

The third ribozyme can target essential factor for DNA biosynthesis, such as DnaG. Every 1 to 2 seconds, at least 1,000 times for each replication fork within *E. coli*, priming of an Okazaki fragment is repeated as a result of an interaction between the cellular primase dnaG (Bouche et al., 1975, J. Biol. Chem. 250:5995-6001) and *dnaB* (Marians, K.J. 1996, Replication Fork Propagation, p. 749-763. In F.C. Neidhardt (ed.), Escherichia coli and Salmonella: Cellular and Molecular Biology, 2nd ed, vol. 1. American Society for Microbiology, Washington, DC.). As would be expected of a protein required every 1 to 2 seconds during replication, a lesion within DnaG or an alteration in its concentration results in an immediate stop phenotype (Marians, K.J. 1996, Replication Fork Propagation, p. 749-763. In F.C. Neidhardt (ed.), Escherichia coli and Salmonella: Cellular and Molecular

Biology, 2nd ed, vol. 1. American Society for Microbiology, Washington, DC.); Weschler et al., 1971, Mol. Gen. Genet. 113:273-284). Therefore, inactivation of the DnaG message by a ribozyme should have profound cellular consequences in that general priming of the lagging strand is reduced if not eliminated. DnaG is a component of the primosome, a multi-protein complex responsible for priming replication. Any of the components of the primosome, either individually or in any combination, can serve as a target for inactivation of the primosome and, thus, kill the cell. The other components of the primosome are *DnaB*, *DnaC*, *DnaT*, *PriA*, *PriB*, and *PriC*. Thus, the primosome is also sufficiently complex to provide numerous other targets (*DnaB*, *DnaC*, *DnaT*, *PriA*, *PriB* and *PriC*) for inactivation by the trans-acting ribozyme.

A fourth target can be *ftsZ*. This gene also encodes an essential protein, *ftsZ*, that is required for cell division in that it is responsible for the initiation of separation. *ftsZ* was selected because cleavage of the *ftsZ* RNA leads to inhibition of cell division and a reduction in viability. Any toxic agent or ribozyme which targets *ftsZ* (such as *DicF1*) may be used to inhibit division of a cell requiring the *ftsZ* gene product. Also, for example, upon cleavage of the *ftsZ* message by a ribozyme, such ribozyme can attack additional copies of the *ftsZ* message inhibiting the division of the cell. The nucleotide sequence of *ftsZ* like the other targets selected, is commonly available from GenBank.

It should be clear that any other essential protein of a pathogen can have its message targeted in the present invention, and that determining which proteins are essential can be routinely determined according to standard protocols in the art. In fact, there are over 52,000 viral, 41,000 bacterial and 12,300 fungal sequences deposited in the public section of the Entrez Database at the National Center for Biotechnology Information. Any of these can be used to design the catalytic trans-acting ribozyme of the invention.

In addition to targeting mRNA of essential proteins, ribozymes may be targeted against other RNA species within the cell. Specifically, appropriate targets in bacteria, fungi and other lower eukaryotes include ribosomal RNA such as Small Subunit RNAs (SSU) or Large Subunit (LSU) and tRNA molecules required for protein synthesis. With respect to pathogenic *Staphylococcus*, the RNA III moiety in a relatively low abundance transcript which is not translated and should be accessible for cleavage. As long as the RNA targeted contains a canonical ribozyme cleavage domain, the ribozyme therapeutic can hybridize and cleave the complementary RNA, thus impacting the fitness of the microbial cell. Additionally, over 3000 rRNA species have been sequenced and aligned. This information is available from the Ribosomal Database Project and should facilitate rapid design and adaptation of ribozyme(s) against such targets. For example the 16S rRNA molecule of bacteria is especially attractive in that there are over 4000 copies of the 16S

rRNA per cell. Consequently, a reduction in number slows the process of protein synthesis in so far as the 16S rRNA molecule is involved in the process of translational initiation. Thus, a toxic agent or ribozyme directed against mRNA and rRNA impacts the fitness of the offending microorganism.

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5.8. PROTECTION OF TOXIC AGENT AND/OR RIBOZYME PRODUCING CELLS

The nucleic acids coding for the toxic agents or ribozymes can be toxic to the cells that are needed to produce the toxic agent or ribozyme-carrying virions. When using a broad host-range virus like P1, the organism used to produce the virion can be different from the target organism. In this way, the producing strain is resistant to the toxic effects of the toxic agents or ribozymes because they are not efficiently expressed in the producing strain, due to species-specific promoter elements, and the ribozymes will not have any target RNA molecules to attack, due to the species-specific sequences that target the ribozymes. When using a species-specific virus that must be expressed and assembled within a strain of the targeted microorganism, this toxicity becomes a significant concern. The assembly of a virion consisting of anti-*E. coli* ribozyme or toxic agent genes packaged in lambda will illustrate the approach used to circumvent the toxicity. For example, the ribozymes directed against RNA species of *E. coli* is expressed from an artificial promoter containing consensus promoter elements. This promoter provides high level transcription of the ribozyme immediately upon infection of targeted cells. In order to prevent the unwanted death of the producing strain of *E. coli*, transcription is repressed in the producing strain by a mechanism not available to the wildtype strains that are targeted for killing. Sequences constituting the DNA binding sites for a heterologous transcription factor are interspersed between the essential activating elements of the ribozyme promoter. Expression of the heterologous transcription factor in the producing strain results in the occlusion of the activating promoter elements and preventing the binding of RNA polymerase. As an example, the gene for the *Saccharomyces cerevisiae* transcription factor Ste12p may be expressed in *E. coli* and bind to its binding sites, the pheromone response element, located within the ribozyme promoter. Ste12p will not be found in wild strains of *E. coli*; therefore, the ribozyme promoter will be accessible to RNA polymerase following delivery of the plasmid to the targeted cells.

An alternative strategy that can protect the producing strain from the toxicity of the ribozymes employs ribozyme-resistant versions of the targeted RNA molecules. This strategy can be used when the target RNA molecule codes for a protein. The ribozyme target site within the mRNA molecule is mutated by site-directed mutagenesis such that the

amino acid sequence of the translated protein does not change but the mRNA sequence no longer serves as a substrate for the ribozyme. For example, hammerhead ribozymes require an NUX sequence within the target mRNA for cleavage to occur. By changing this sequence to something else, the ribozyme will not cleave the mRNA. This type of ribozyme
5 resistant version of the target RNA can be expressed from a plasmid or integrated into the chromosome of the producing strain and thus render this strain resistant to the toxic effects of the ribozyme.

Another strategy that can protect the producing cell from the toxicity of a toxic agent employs co-expression of a neutralizing agent or antidote. Such co-expression
10 of an antidote or neutralization agent protects the packaging cell from the toxic effects of the encoded toxic agent. Such a strategy is particularly useful is the promoter used to express the toxic agent is leaky, and leads to expression of the toxic agent in the producing cell. For example, a packaging strain (*e.g.*, bacteria cell) may used to package the a viral vector containing a toxic agent into a bacteriophage phage head. Survival of the packaging
15 cell or optimization of the quantities of vector or phage made by the producing cell may require co-expression of an antidote or neutralization agent in the producing cell. A neutralization agent is any molecule (such as protein, antisense, sense, or other molecule (such as a drug, chemical, etc.)) which counteracts the toxic effects of a toxic agent. By way of illustration, in a specific example, the packaging strain cells contain a bacteriophage
20 P1 plasmid as well as the Transfer plasmid comprising the toxic agent *doc* and a ribosome entry site. In the case that the Transfer plasmid is determined to be toxic to the packaging strain, a third plasmid may be introduced, which encodes an antidote to *doc*, such as the *phd* protein. The additional plasmid with the antidote acts to protect the packaging strain against the toxicity of *doc*.

25 The improvement in the present invention is that a non-replicative delivery system has an advantage in that once the phage coat has injected the nucleic acid into the targeted bacterium, the expression of the toxic agent or ribozyme will destroy the microbe, as opposed to a lytic infection cycle typical of an intact bacteriophage. Consequently, amplification of the phage coat will not be an issue and it is less likely that the non-
30 replicative phage delivery system will generate an immune response such that subsequent use of the delivery system would be jeopardized. Moreover, if the patient has been exposed to a resistant pathogenic microbe and the therapeutic of the invention is effective and neutralizes the invading microbe, then it is expected that the microbial antigens liberated as a result of the action of the therapeutic, will illicit sufficient humoral immunity and cell-
35 mediated immunity to confer protection against subsequent attacks.

5.9. THERAPEUTICS & PHARMACEUTICAL PREPARATIONS/FORMULATIONS AND METHODS FOR ADMINISTRATION

5 The present invention further encompasses the use of a toxic agent and/or
ribozymes of the present invention for the treatment of disease, viral infection, parasitic
infection and microbial infection. The present invention further provides a method of
treating a subject having a proliferative disease of a specific tissue by inhibiting cell
proliferation in the tissue, comprising administering to the subject a toxic agent and/or
ribozyme operably linked to a tissue-specific promoter sequence, which promoter is specific
10 for the diseased tissue, and whereby the ribozyme and/or toxic agent encoded by the nucleic
acid is expressed, cell proliferation is inhibited, and the proliferative disease is treated.

The present invention further provides a method of treating a subject having
a pathogenic infection or disease, by inhibiting replication of the pathogen, comprising
administering to the subject a toxic agent and/or ribozyme operably linked to a pathogen-
15 specific promoter, whereby the ribozyme and/or toxic agent encoded by the nucleic acid is
expressed, the pathogen is inhibited from replicating or is killed or rendered less fit, and the
infection or disease is treated. The present invention encompasses the toxic agent(s) and/or
ribozyme(s) of the present invention in pharmaceutical formulations.

In several embodiments of the invention, toxic agents or ribozymes of the
20 invention are particularly suited as antimicrobial therapeutics. For example, upon nucleic
acid hybridization with the target RNA transcript, a ribozyme-RNA complex achieves a
catalytic form that acts as a nuclease to cleave the targeted RNAs. Thus, cleavage deprives
the invading microorganism of essential cellular processes which then kills or renders it less
fit. Additionally, a toxic agent of the invention may also be used as an antimicrobial
25 therapeutic. A toxic agent may be used alone, or in combination with one or more other
toxic agents. Thus, delivery of a toxic agent to an invading microorganism, kills or render it
less fit. A toxic agent may also be used in combination with one or more ribozymes.
Further, a combination of ribozymes and toxic agents may be used as an antimicrobial
therapeutic.

30 The invention provides use of one or more ribozymes and/or toxic agents
directed towards essential, housekeeping, or virulence genes of one or a series of candidate
microorganisms. Inactivation of essential proteins and virulence determinants render the
invading microbes inactive or slow their growth, while at the same time, the essential
processes of the host are not significantly affected.

35 A method of delivering a toxic agent or ribozyme to a target (*e.g.*, a
pathogen) in a subject is provided, comprising a) generating a liposome comprising a

promoter and a sequence encoding a toxic agent or ribozyme; and b) delivering the liposome to the subject, whereby the target-specific promoter directs transcription of the toxic agent or ribozyme in the cells of the target. The target can be a pathogen, for example, a bacteria, fungus, yeast, parasite, virus or non-viral pathogen.

5 A method of targeted delivery of a toxic agent or ribozyme to a pathogen in a subject is provided, comprising a) generating a virion comprising non-viral DNA of the invention; b) combining it with a liposome; and b) delivering the liposome containing the virion to the subject, whereby liposome enters the eukaryotic cell and releases the virion, which delivers the DNA to the pathogen, whereby the pathogen-specific promoter directs
10 transcription of the toxic agent or ribozyme in the cells of the pathogen.

 A method of treating an infection in a subject is provided, comprising administering to the subject the liposome comprising DNA comprising a target-specific promoter and a sequence encoding a toxic agent or ribozyme, whereby the toxic agent or ribozyme encoded by the DNA is expressed and the infectious agent is killed or weakened.
15 The liposome used in this method can comprise any ribozyme-encoding nucleic acid, or any toxic agent-encoding nucleic acid, particularly those described herein targeted to genes of the pathogen. The infection can be bacterial, fungal, yeast, parasitic, viral or non-viral.

 Parenteral administration, if used, is generally characterized by injection (intravenous, intradermal, subcutaneous and intramuscular). Injectables can be prepared in
20 conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant level of dosage is maintained. *See, e.g.*, U.S. Patent No. 3,610,795, which is incorporated by reference herein. In certain preferred embodiments of
25 the invention administration is parenteral.

 The present invention relates to prophylactic administration. For example, many hospital patients or immunocompromised hosts are particularly susceptible to pathogenic infections. Further, many hospital strains of pathogens are resistant to traditional antibiotic treatment, such as Penicillin. The therapeutics of the invention are
30 particularly useful for preventing pathogenic infection or treating infections caused by resistant strains of pathogens.

 Suitable carriers for parenteral administration of the substance in a sterile solution or suspension can include sterile saline that can contain additives, such as ethyl oleate or isopropyl myristate, and can be injected, for example, intravenously, as well as
35 into subcutaneous or intramuscular tissues.

Topical administration can be by creams, gels, suppositories, aerosols, sprays, and the like. *Ex vivo* (extracorporeal) delivery can be as typically used in other contexts. In certain preferred embodiments of the invention administration is an aerosol. In other preferred embodiments of the invention, administration is as a topical treatment. In one embodiment of the invention, treatment of infections associated with burns or open wounds, topical administration may be preferred.

Oral administration is also provided. Suitable carriers for oral administration include one or more substances which can also act as flavoring agents, lubricants, suspending agents, or as protectants. Suitable solid carriers include calcium phosphate, calcium carbonate, magnesium stearate, sugars, starch, gelatin, cellulose, carboxypolymethylene, or cyclodextrins. Suitable liquid carriers can be water, pyrogen free saline, pharmaceutically accepted oils, or a mixture of any of these. The liquid can also contain other suitable pharmaceutical additions such as buffers, preservatives, flavoring agents, viscosity or osmo-regulators, stabilizers or suspending agents. Examples of suitable liquid carriers include water with or without various additives, including carboxypolymethylene as a pH-regulated gel.

The therapeutic of the invention can be administered to the subject in amounts sufficient to produce an antibiotic effect or to inhibit or reduce the activity of the target pathogen. Optimal dosages used will vary according to the individual, on the basis of age, size, weight, condition, etc, as well as the particular modulating effect being induced. One skilled in the art will realize that dosages are best optimized by the practicing physician and methods determining dosage are described, for example, in Remington's Pharmaceutical Sciences [Martin, E.W. (ed.) Remington's Pharmaceutical Sciences, latest edition Mack Publishing Co., Easton, PA.]. Treatment can be at intervals and can be continued for an indefinite period of time, as indicated by monitoring of the signs, symptoms and clinical parameters associated with a particular infection. The parameters associated with infection are well known for many pathogens and can be routinely assessed during the course treatment.

6. EXAMPLE: CONSTRUCTION & CHARACTERIZATION OF PROMOTERS

Classical bacterial inducible promoters are renowned for their inability to tightly control transcription, and a significant level of background expression is characteristically observed.

The Leashi Promoter

The present invention provides bacterial promoters that allow for tight regulation of transcription and enhanced expression. A novel promoter called LEASHI has been constructed from three elements (see Figure 1A). The first element, termed RIP was a combination of two consensus sites at -10(TATAAT) and -35(TTGACA) located with respect to transcription initiation. The second element was based on the lacI repressor binding sequence (termed lac operator sequence) which was placed between the -10 and -35 consensus sites. Placement of the lac operator between the -10 and -35 sites, more effectively blocked RNA polymerase binding to the promoter, thus enhancing transcriptional control from the promoter. The promoter was designed such that it was 'switched on' following the addition of isopropyl B-D-thiogalacto pyranoside, which binds and subsequently titrates out the repressor protein. RNA polymerase can then bind to the promoter and transcription can proceed.

The third element of the LEASHI promoter was the UP element. The UP element was an adenine/thymine rich sequence which was placed immediately upstream of the -35 element. Addition of the UP element, further increased expression from this promoter.

The LEASHI promoter sequence: (SEQ ID NO:1)

5' GATCCTCAGAAAATTATTTTAAATTTCCAATTGACATT
GTGAGCGGATAACAATATAATGTGTGGA3'

A significant advantage of the LEASHI promoter of the present invention is that it alleviates the high levels of background commonly observed in inducible promoters. A limiting factor leading to high background levels of transcription when a promoter of interest is located on a high-copy number plasmid, is due to the lack of repressor molecules available to bind to the promoters. The present invention overcomes this problem by using a lacI expression plasmid and secondly, by placement of the lac operator between the -35 and -10 consensus elements which more effectively blocks transcription during normal conditions. Furthermore, the UP element placed immediately upstream of the -35 region enhanced transcription from the promoter.

The LEASH1 promoter (Figure 1B) was designed as a lacI-regulated promoter which has a broad spectrum promoter activity in a wide variety of bacteria. The IPTG inducible LEASH1 functions in *Escherichia coli* and is tightly regulated. It is active in both Gram-negative and Gram-positive bacteria.

As described herein, ribozymes of the invention have been operably linked to the LEASHI promoter. In another specific embodiment of the invention, a toxic agent of the invention was operably linked to a LEASHI promoter.

5 ***The Modified rrnB Promoter***

A novel promoter called the modified rrnB has been constructed (see Figure 1C).
Modified rrnB promoter sequence: (SEQ ID NO:2)

10 5'AGAAAGCAAAAATAAATGCTTGACACTGTAGCGGGAAGGCGTATA
ATGGAATTGTGAGCGGATAACAATTCACA 3'

The Anr , Arc, and Proc Promoters

The *Anr* (Figure 1D), *proC* (Figure 1E) and *Arc* (Figure 1F) promoters are
15 species-specific. Both *anr* and *proC* are transcriptionally off in *E. coli* and on in
Pseudomonas aeruginosa. These promoters provide the advantage of allowing controlled
expression of the toxic agents in the pathogen (*Pseudomonas*), while allowing the packaging
strain (*E. coli*) to be protected from the toxic actions of the therapeutic. Such promoters are
particularly useful to facilitate manufacturing of the delivery vehicle. Such promoters also
20 enable bacterial specific targeting of the gene therapeutic in the patient.

Pseudomonas aeruginosa 'specific' promoters (5' to 3')

ANR promoter (SEQ ID NO:3)

5' ACTCGCGGATCATCTTCACCATCGGCCGCAACTCCTGCGGGATATCCTCGTC
25 CTCCTCCTCCACCGGCACCCCATGGTAGCGGCCAGCTCGCGCCCTGCCTGGGA
AAGCTGTACATGCTGATCGGCGGCGTCGGTGCCGGCGGCCGGGTCTTCCGCCTG
CTCGGCGGTGCCGGTCCGTGCGGCCTTGGCGTCCGCGGCGGCGCGCATGAGGG
CGGCACCTGGGTGGTGATCCAGCCACTGAGGGTCAACATTCCAGTCACTCCGGG
AAAAATGGAATTCTTCCATTGGATCGGCCACGCGTCGCGAACTTGAGCCCCCT
30 TTTCGTCGCCCCTTGACAGGGTGCGACAGGTAGTCGCAGTTGTTTGACGCAAGT
CACTGATTGGAAACGCCATCGGCCTGTCAGAAATGGTCGTTGCCAGACCTATGG
CTGGCACCCGCATCGCGGCTGCGTTACCCTTACTCCTGTTGTGCCTTTAACCTAG
CAAGGAC

35

ProC promoter (SEQ ID NO:4)

5' AATTCCTCGAAGTCCTTGCGCTGCTTGTCGTTTCATGATGTCTAGATCAGCGC
ATGCACCTGCTTGTGTTCCAGCGGTGGCAGGTTGATCCGGCGTACATCGCCATC
CACCCGGATCATGGGTGGCAGGCCGGCGGAGAGGTGCAGGTCCGAAGCGCCCT
5 GTTTGGCACTGAAGGCGAGCAGCTCGGTAATATCCATGGGACTCCCCAATTACA
AGCAAGCAGGTAGAAATGCCGCCAAAGCCGCCGTCTCGGACAAGGAAAACACCG
GATGAGCCAGGGTGCTTCCAGGACACGCGTGGTGTCTGCGCCAGACGCGGAA
CCTCGACACTGGAACAGGAAGATGGCCATCGAGGCCGGCGGTTTCGAGGGCGT
CGAGCCGACGCCGACCGCACTTCCATAGGGCGCAGGTAATGTCCACGATAGCA
10 GAGAATATTGCAAAGGTTGCCGCGCGCATCCGTGAGGCAGCGCAAGCTGCGGG
GCGCGATCCGGCCACGGTCGGCCTGCTCGCCGTGAGCAAGACCAAGCCCGCCG
CCGCGGTGCGCGAGGCGCACGCCGCCGGCCTTCGCGACTTCGGCGAAAACCTACC
TGCAGGAGGCCCTCGGCAAGCAGGCCGAACCTGGCCGACCTGCCCTTGAACCTGG
CACTTCATCGGCCCCATCCAGTCGAACAAGACGCGGCCCATCGCCGAGCA.TTTC
15 CAGTGGGTGCACTCGGTGGACCGGTTGAAGATCGCGCAGCGCCTGTCTGGAGCA
ACGCCCCGGCCGGGCTGCCGCCCTGAATGTCTGCCTGCAGGTCAACGTCAGCGG
CGAAGCCAGCAAGTCCGGCTGCGCCCCCGAGGACCTGCCGGCCCTGGCCGAGG
CCGTGAAGCAACTGCCCAACCTCCGATTGCGTGGCCTGATGGCCATCCCCGAAC
CCACCGCCGAACGCGCCGCGCAACACGCCGCGTTCCGCCGCCTGCGCGAACTG
20 CTGCTGGACCTGAACCTTGGCCTGGACACCCTGTCCATGGGCATGAGCGACGAC
CTCGAGGCAGCCATCGGCGAAGGTGCGACCTGGGTCCGCATCGGTACCGCCCTG
TTCGGCGCCCCGCGACTACGGCGCGCCGGCTTCTTGAATGAATCCC

ARC promoter (SEQ ID NO:5)

25 5' CTA GAG CTA TTG ATG TGG ATC AAC ATT GTC CAC TAG CCG CTG
CCG CCT AAT CTC CAG AAT TGT GAG

Anr, *Arc*, and *proC* promoters, which were expressed preferentially in *P. aeruginosa*, have been isolated and shown to express a toxic agent specifically in this
30 pathogenic bacterium (See Tables 1 and 2 and Figure 2). Specifically, as shown in Table 1, promoters were cloned upstream of the β -lactamase reporter gene in a cassette flanked by multiple transcription terminators. Constructs were transformed into *E. coli* or *P. aeruginosa* and plated onto agar containing different amounts of carbenicillin. Three repeat evaluations gave the same result.

35

Table 1. Evaluation of Promoters Utilizing β -Lactamase as a Reporter Gene

Construct	E. coli		P. aeruginosa
	25 μ g/ml carbenicillin	50 μ g/ml Kanamycin	5 mg/ml carbenicillin
empty vector (no promoter)	-	+	-
UPRIP β -lactamase	+	+	+
<i>proC</i> β -lactamase	-	+	+
<i>anr</i> β -lactamase	-	+	+

As shown in Table 2, the *chpBK* gene was cloned in both orientations under the control of *P. aeruginosa* promoters *proC* and *anr*. Equal quantities of DNA (500 ng) were transformed into *E. coli* and *P. aeruginosa* and plated on agar. Mock transformations were also performed with 'no DNA'. + indicates greater than 100 colonies, - indicates no detectable colonies. Parentheses indicate orientation of the *chpBK* gene in relation to the promoter. Experiments were repeated at least two times with the same result. Importantly, plasmids using *proC* and *anr* to regulate *chpBK* expression did not induce cell death in *E. coli* indicating lack of transcriptional activation function.

Table 2. Evaluation of Promoters that are Expressed Preferentially in *P. aeruginosa*

Construct	<i>E. coli</i>	<i>P. aeruginosa</i>
patent vector	+	+
<i>anr chpBK</i> (positive)	+	-
<i>anr chpBK</i> (negative)	+	+
<i>proC chpBK</i> (positive)	+	-
<i>proC chpBK</i> (negative)	+	+

The development of species-specific promoters is particularly important in aspects of the invention in which it is desired to allow indigenous commensal bacteria to be protected from the toxic agents of the invention while targeting the pathogenic *P. aeruginosa*.

TSST-1 Promoter

The environmentally regulated staphylococcus-specific promoter TSST-1 has been obtained and a transfer plasmid utilizing this promoter is used to express *doc* or other toxic agents. A staphylococcus specific phage capable of delivering the transfer plasmid into *S. aureus* strains is used to specifically target the Staphylococcal pathogen.

TSST-I promoter (SEQ ID NO:6) (GenBank accession number U93688, see also Lindsay, J.A., et al., 1998, "The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*" Mol. Microbiol. 29 (2), 527-543):

10

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1  ttatttagca ggaataatta gccagattat cgagggagtt ccagggcaatccaaacattg
61  ttatatatgc attataaaa tttcaagat aatttattat tcatacccttgcccttgtt
121 tcaaaattat gccctttttt tgcccttgga aacaaccaca ctctaaattaataggtggt
181 gtggtttgat catttataat ataacataaa aacaaccacc cagtaactagtagtggtggc
241 gtagcgacta taacaactct atgttatcaa gatatatgta tatgagtgatgacaaggaag
301 atgtctcttg tgagaccaac agccagatat atggcctctt gccgggctatatagttcact
361 cctactatat acacatgtaa ttataacata aaaaaataga caagtaccgaagtacctgcc
421 taaataacaa caagattaac atgtgaataa tggaaataaa aagtcagcccgaaggctaac
481 ttacgaatag atgaaaattt gaacacattg ctgtgtctaa aatgattatagcataataa
541 cgaatatttc cagctcgaaa ttaatatatt gtaataataa tattttatatctttgttaat
601 aattatttaa ttgatttaca taaataataa ttgtaaaatt aatttgtaatcgattgcaaa
661 taagtatatg gagaaaataa aatgaataaa aaactattaa caaaaacattgatagcaagt
721 gctttagttt taacaacagt aggttcaggt ttcatctctt cttcaaattataatggtatt
781 aataacgttg aaaaagctga gcaaacgaca gataacgcat tgtggaaaaatgtaagagac
841 gctttaaag acgcgaatat tatcgataaa acagataatg aaaatgtcaagggtacgtat
901 aaaatagaaa atggtggaga aaataccata gaaggaacag ttaatttagaaaatattagt
961 acttcaaaca atcctaaaat aaacctcaa aatgttaca aaattaatataactagaaaa
1021 aatccgaact accetaatat tgatgctaata aatacatgga aaaaattaccagaaaaattg
1081 aaagaaaaaa atatagtgga acaacggcga caatgtttca atcttaagtacagaccctaa
1141 agatgagact gtattcggtg aagtaggaga agataaatca aacgtaagcaatagatacat
1201 caatcctaaa gatataaatg aattcaaatc actaaaaata ctttttccgaggcagatta
1261 ctctgcctc tttcttgaa cagtgatatc ttctgatcta tgtaacactcaattactca
1321 gattctttac cttaacttc cttaattca tttctctcta tctctcaaaaagttgtgct
1381 ttttgatttg tgattggagt tgggcgtttt tcatcgcggt tgttcaattccttttaag
1441 gtattctaata tctctctag tcatatcaat tgtttttta ctctcaccttagtgaaat
1501 actcttatcc tttctctct tgcgttaatg ttgctaatta gtataaaatcatgcgcccc
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35

1561 tatattccaa tggtaggaca ttaattctg gattttcagc tattttcataaatctattat
 1621 ctgataattt gcttaatcca attttcaagc catagcctaa attccccatccactaagtc
 1681 ttttgttca tatggttta atctacggcc aatctcaaag atagattgaccagcgatgtt
 1741 taaagtcata tttcacggat ccacatttac gataaacata tctagttacacaatattatc
 5 1801 ccttactgca acacaggacg ttctcagcg taaaaaacac cactagaaagtgcatttaaa
 1861 gaataataact aattcaaact tatattaatt aatattcttt aaatgaccactcacactttg
 1921 tttttgcta ttgtactt taaaatgttg ttgaaatct atattttttgatatagctc
 1981 cctatgtaac aaacaatttt taattaatat atatttaaac aagtcaatttagagatcggt
 2041 taattcgatt catttaaata atatttatac attctatatg taaacgtttacacatttgaa
 10 2101 gtaaggagaa ttaaaaatga

7. EXAMPLE: EFFECTS OF TOXIC AGENTS ON BACTERIAL GROWTH

15 In order to demonstrate the methods of the invention, the inventors have expressed and targeted several toxic agents to bacterial pathogens. Toxic agents were selected based on their ability to inhibit the growth of a pathogen or diseased cell or cause lethality in a pathogen or diseased cell. The examples hereinbelow illustrate toxic agents of several naturally occurring phage, plasmid and chromosomally encoded toxic proteins and
 20 demonstrated their effectiveness as antimicrobial therapeutic agents.

Specifically, several naturally occurring phage, plasmid and chromosomally encoded toxic proteins have been identified and have demonstrated effective as antimicrobial therapeutic agents, including but not limited to *SecA*, *16S RNA*, *dicF*, *sof*, *dicF* antisense, *16S* antisense, toxic proteins of the toxin/antidote pairs *doc/Phd*, *gef/Sof*,
 25 *chpBK/ChpB1*, or *kicB/KicA*.

To illustrate that a toxic agent may be a toxic gene product of an addiction system toxin, a toxic gene product of a chromosomally encoded toxin, or antisense molecule, nucleic acids encoding *doc*, *gef*, *chpBK*, *kicB* or *DicF1* were engineered into Transfer plasmids for use in the P1 bacteriophage delivery system. Plasmid construction
 30 was performed by standard methods known in the art.

As shown in Figure 3, expression vectors for the cloning of Toxic agents were engineered. Specifically, genes encoding the toxic proteins *chpBK*, *kicB*, *doc* and *gef* under the control of the *lacI*-regulated promoter, were cloned into an *E.coli* vector containing replication origin *ColE1* (300-500 copies per cell), *pMB1* (15-20 copies per cell)
 35 or *p15A* (10-12 copies per cell) and the selectable marker *cat*., chloramphenicol or *bla*, ampicillin. Toxic agents were cloned in an *E. coli* strain that overexpressed the *lacI*

repressor protein from a *lacI* expression plasmid. Genes encoding the toxic proteins *chpBK*, *kicB*, *doc* and *gef* under the control of *lacI*- regulated promoter, were obtained by PCR and cloned into an *E. coli* shuttle vector. Lethal agents were cloned in an *E.coli* strain that overexpressed the *lacI* repressor protein form a *lacI* expression plasmid.

5 Plasmids containing the toxic agents *doc* or *gef* were also been engineered such that a ribosome entry site has been constructed upstream of the nucleic acids encoding the toxic agent in order to increase the levels of translation of *doc* or *gef*. Plasmids harboring a toxic agent was called a Transfer plasmid. The Transfer plasmid was constructed such that it contained 1) an origin of replication 2) selectable marker 3) P1 PAC
10 site, and PAC ABC genes 4) P1 lytic replicon 5) nucleic acids encoding the toxic agent (*e.g.*, *doc*, *gef*, or *DicF1*).

Specifically, Transfer plasmids were constructed based on pBluescript (ColE1 origin) and pBBR 122 (broad host range origin) parent vectors. The nucleic acids encoding the toxic agents *doc* or *gef* were cloned into the broad host range transfer plasmid.
15 The nucleic acid encoding *dicF* was cloned into the ColE1 transfer plasmid. The structure of each vector is available. Both *doc* and *gef* were placed under *lacI* regulated promoter. The Transfer plasmids were designed to undergo rolling circle replication during the phage lytic cycle.

A packaging strain (*e.g.*, bacteria cell) was then used to package the Transfer
20 plasmid containing the nucleic acid encoding the toxic agent into a bacteriophage phage head. The packaging strain for each of the three toxic agents contained the P1 bacteriophage prophage as well as the Transfer plasmid containing the nucleic acids encoding the toxic agent. In some cases, the packaging strain also contained a third plasmid, if necessary, which encoded additional antidote protein which acted to protect the packaging strain
25 against the toxicity of the toxic agent or the third plasmid encoded additional repressor protein to switch off the promoter of the Transfer plasmid.

Thus, the packaging strain (P1 lysogen) was used to package the transfer plasmid containing the toxic agent (*e.g.*, *doc*, *gef*, or *DicF1*) into phage heads or virions. Phage lysates of the packaging strain contained the infectious bacteriophage virions, and
30 were used to infect bacterial targets in the following manner.

The P1 lysogen (P1cm C1.100) carrying the transfer plasmid with the toxic agent (*doc* or *gef* or *DicF1*) was grown at 30°C in LB, 10 mM MgSO₄, 5 mM CaCl₂, 12.5 µ/ml chloramphenicol unit A₄₅₀ reached 0.8 at which time the culture was shifted to a 42°C water bath and aerated vigorously for 1 h. Chloroform was added and incubation continued
35 for an additional 20 min at 37°C. The phage stock was clarified by centrifugation at 4,000 g for 20 min. DNase (1 µg/ml) and RNase (10 µg/ml) were added and after incubation at

37°C 30 min the phage were centrifuged at 4,000 g 20 min. Phage particles were precipitated from the phage stocks by adding NaCl to 1 M and polyethylene glycol 6000 to 10% (w/v). After incubation on ice for 2 h the phage were pelleted by centrifugation at 11,000 g for 15 min. The pellet was carefully dissolved in 50 mM Tris.Cl pH 7.5, 10 mM MgSO₄, 5 mM CaCl₂, 0.01% Gelatin. Polyethylene glycol was removed by extraction's with chloroform.

The phage lysates were then used to infect a selected pathogen (*e.g.*, *E. coli*). Target cells (10⁵ CFU/ml, treated with 10 mM MgSO₄, 5 mM CaCl₂) were infected at various M.O.I s (0.1, 1, 10, 100) with each of the above phage lysate. Following 30 min infection at 30°C. Cell death was assessed by scoring the plates for the total number of colony forming units.

Both types of Transfer plasmids (ColE1 and broad host range based) were transferred by the P1 delivery system to various *E. coli* strains *in vitro*. The P1 system was also used to deliver the broad host range traTsfer plasmid to *P. aeruginosa* *in vitro*. The ColE1 transfer plasmid was successfully transferred to *E. coli* *in vivo* and the broad host range transfer plasmid has been delivered *in vivo* to both *P. aeruginosa* and *E. coli*.

Results indicated that the infection of the bacterial cells with the phage lysates comprising the infectious virions containing a toxic agent was capable of killing the infected bacterial cells. Further, bacterial cell death was seen to be dose dependent such that higher M.O.I lead to increased cell death. Thus, the methods and compositions of the invention are useful as antimicrobial agents for treating pathogenic infections.

Lethality testing of the toxic agents and has revealed that *doc*, *gef*, *chpBK* and *kicB* are all bactericidal to *E. coli*. (See Figure 4). Specifically, colonies were grown in liquid culture under conditions where the expression of the toxic proteins was repressed. following expression of the protein by induction with IPTG for 1 hour, cultures were plated out overnight onto agar lacking IPTG. The absence of colonies indicates the protein is lethal (see also Table 4 for Results). Constructs were transformed into *E.coli* and plated onto agar with or without 1mM IPTG. Equal quantities of DNA (500 ng) were also transformed into *P. aeruginosa*, *S. aureus* and *E. faecalis*. Mock transformations were also performed with 'no DNA.' + indicates greater than 100 colonies, - indicates no detectable colonies. Experiments were repeated at least two times with the same result. All agents were lethal to *E.coli* but only *doc* was toxic in all four.

Table 4. Assessment of the Toxic Proteins in *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* using the Broad Host Range Plasmid.

Construct	<i>E. coli</i>	<i>E. coli</i> +IPTG	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>E. faecalis</i>
Parent vector	+	+	+	+	+
<i>doc</i>	+	-	-	-	-
<i>gef</i>	+	-	+	+	+
<i>chpBK</i>	+	-	-	+	+
<i>kicB</i>	+	-	+	+	+

As shown in Figure 5, the growth of *E. coli* harboring a *doc* Expression plasmid was demonstrated to be inhibited when the expression of *doc* was induced by IPTG. Specifically, cells were grown overnight in LB at 32°C, diluted 1:100 into fresh LB medium and incubated at 32°C for 180 min. The culture was then divided equally and incubated at 32° in the absence (○) or presence of 2 mM IPTG (○) which results in the expression of the lethal agent *doc*. Growth was calculated by spectrophotometric measurements with 1 ml samples at OD600.

Unlike traditional antibiotics which merely slow the growth of the bacteria, lethality testing of *doc* has shown that 99% cell death was achieved when cells were induced with IPTG for 20 min. A significant reduction (92% cell death) was also demonstrated when the cells were under no selective pressure to maintain the *doc* expression plasmid. Thus, the rapid killing of bacteria reduces the potential for selective pressure to give rise to resistant strains, which is important in eradicating multidrug resistant bacteria.

LOW RESISTANCE TO *DOC*

In order to examine the frequency of resistance to *doc*, resistant mutants were isolated. The rationale was to select for spontaneous mutations and no mutagens were used. Following prolonged exposure to sublethal concentrations of 40 *doc* resistant *E. coli* clones were isolated. DNA isolated from these clones were tested by transformation to a *doc*-sensitive cell, however the presence of IPTG did not induce cell killing. This indicates that resistance is due to mutations or recombination events in the *doc* expression plasmid, suggesting that a chromosomal mutation of the *doc* target occurs at a very low frequency.

TOXICITY TO *P. AERUGINOSA*

Doc and *chpBK* have been demonstrated to be toxic to *P. aeruginosa*. Of particular note is that *doc* had a broad-spectrum activity in both Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *E. faecalis*) bacteria (see Table 4, above).

- 5 As seen in Table 4, the toxic agent *doc* killed all species of bacteria tested. *Doc*, *gef*, *chpBK* and *kicB* were all able to kill *E.coli*. *chpBK* killed *E.coli* and *P. aeruginosa*.

DEVELOPMENT OF A BACTERIOPHAGE TOXIC AGENT DELIVERY SYSTEM

- 10 A toxic agent delivery system has been achieved for the use of a bacteriophage P1 system to package and deliver a Transfer plasmid (See Figure 6A and 6B) to *E. coli* and *P. aeruginosa*. Figure 6A depicts the Transfer plasmid containing the essential signals for packaging (a *pac* site and a lytic replicon under the control of the P1 P53 promoter), a selectable marker for detection (*bla*, ampicillin) and ColE1 origin of
15 replication in *E. Coli*. Figure 6B depicts the lytic replicon which comprises the C1 repressor-controlled P53 promoter antisense and genes *kilA* and *repL*. The *kilA* gene contains a 52% in frame deletion. P53 antisense is implicated in the stability of the P1 replicon. The methods of the invention are exemplified herein by two transfer plasmids capable of being efficiently packaged in P1 virions for delivery to pathogenic Gram-
20 negative bacteria. Importantly, the delivery system is not under the constraints of superinfection exclusion (Figure 7). In order to demonstrate delivery efficiency of the Transfer plasmid by the P1 Delivery System to *E. coli*, the following assay was performed. The *E. coli* P1Cm clts100 lysogen carrying the transfer plasmid was induced by thermal induction to produce phage particles. Phage lysates were created with DNase and RNase
25 and precipitated particles were resuspended in 50 mM Tris.C1 pH 7.5, 10mM MgSO₄, 5 mM CaCl₂, 0.11% gelatin, *E. coli* C600 and *E. coli* P1 C600 target cells (10⁵ CFU/ml, treated with 10mM MgSO₄, 5mM CaCl₂) were infected with each of the phage lysates. Following 30 min incubation at 30°C, infections were plated onto selection plates and antibiotic resistant colonies were scored. Values indicate number of antibiotic resistant colonies ±
30 standard error, n=6.

Further, the phage-based delivery system is not blocked by a resident phage, such as P1 and lambda, or by compatible plasmids. This is important because analyses of environmental samples suggests that up to 40% of *P. aeruginosa* strains in the natural ecosystems (lake water, sediment, soil and sewage) contain DNA sequences homologous to
35 phage genomes. The bacteriophage based system is useful to transfer genetic information *in vivo* by delivery of a transfer plasmid expressing an antibiotic marker to *E. coli* and *P.*

aeruginosa in a mouse peritonitis model of infection. Plasmid transfer was confirmed by restriction analysis and sequencing of the plasmid DNA re-isolated from bacteria recovered from the intraperitoneal space. Demonstration of transfer *in vivo* has also been obtained.

DEVELOP BACTERIOPHAGE P1 KNOCKOUTS ABLE TO PACKAGE TRANSFER PLASMID DNA BUT UNABLE TO INCORPORATE P1 DNA

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10

One consideration of using unmodified phage as a delivery vehicle is the potential risk of lysogenic conversion. In order to develop a bacteriophage delivery vector which is capable of delivering a Transfer plasmid, to a target bacteria, but which is unable to deliver its own DNA to a target bacteria, a modified P1 phage was developed.

15

As shown in Figure 8, the P1 prophage DNA has been modified to generate a *pac* site knockout. The disruption cassette contain a nutritional or antibiotic marker flanked by sequences homologous to the P1 prophage. the linear fragment was protected from exonuclease attack by the incorporation of phosphorothioate groups. A double crossover event between the *in vitro*-altered sequence and the P1 prophage resulted in deletion of the *pac* site and acquisition of the selectable marker. The function of this knockout serves to inhibit the ability of the p1 bacteriophage to package or transfer its own DNA to a target bacteria.

20

25

As shown in Figure 9, the modified P1 was unable to transfer the chloramphenicol marker associated with its genome, suggesting that phage particles produced from the *pac* mutants lack phage DNA. The top panel of Figure 9 shows the physical map of the P1 prophage and predicted P1 knockout following integration of the disruption cassette at the *pac* site. Arrows indicate location of the PCR primers used to verify the replacement of the P1 *pac* site with the *S. cerevisiae* TRP1 gene. The gels shown the products of the PCRs using P1 specific primers (1, 3, 5 and 6) and disruption cassette specific primers (2 and 4) to detect either the wild type P1 prophage or the P1 knockout. Primers 1 and 3 do not bind within the P1 sequences in the disruption cassette therefore PCR with primer 1+2 and 3+4 only detect a specific integration event which results in replacement of the *pac* site with the *S. cerevisiae* TRP1 gene.

30

As a consequence of the *pac* site lying within the *pacABC* operon, the modified phage needed to be complemented *in trans* with the *pacase* enzyme. Construction of the *pacABC* complementing plasmid is shown in (Figure 10 and Table 5).

35

Table 5 Construction of the *pacABC* complementing plasmid.

	c1-pBSK
	clts100-pBSK
	Bof-pACYC184
5	pEDI-clts100-pBSI
	C1pro-clts100-pBSK
	clmut-clts100-pBSK
	Bof-pEDI clts100-pACYC184
10	Bof-C1mut clts100-pACTC184
	Bof-C1pro clts100-pACYC184
	TpacABCT-Bof-pEDI clts100-pACYC184
	TpacABCT-Bof-C1pro clts100-pACYC184
	Tpr94pacABCT Bof-pEDI clts100-pACYC184
15	cl, cl repressor; clts100, C1 repressor with temperature sensitive cl. 100 mutation; Bof, modulator of C1 repressor; pEDI, promoter; c1pro, promoter with C1 operator sites Op99a and Op99b; c1mut, promoter C1pro with mutated operator sites; pacABC, genes encoding pacase enzyme; T, transcrip
20	
	tion terminatore; Pr94, promoter with operator site Op94; pBSK, <i>E. coli</i> vector pBluescript, pSACYC184, <i>E. coli</i> cloning vector.

P1 *pacABC* were expressed from an early promoter Pr94. Two phage encoded proteins, C1 repressor and Bof modulator, were used to regulate expression from the Pr94 promoter. Although Bof alone does not bind to DNA, together with C1 it increased the efficiency of the repressor-operator interaction. The c1 repressor has the clts 100 mutation and was therefore be temperature sensitive. This allowed the coordinated expression during the phage lytic cycle to the *pacABC* genes.

The complementation plasmid allowed P1 *pac* mutant to package the Transfer plasmid but not its own viral DNA. Complementation with the pacase enzymes did allow the P1 *pac* mutants to package the transfer plasmid, however a portion of the phage particles produced from the *pac* mutants contained P1 viral DNA. Analysis of the chloramphenicol resistant transductants indicated that the majority were unable to produce a second round of multiplication, suggesting that they were defective lysogens. The *pac* mutants appeared to have acquired a *pac* site, by recombination with the complementing plasmid, thereby enabling the mutants to package and deliver its own viral DNA.

Southern blot analysis verified that the *pacABC* genes on the complementing plasmid had been replaced with the *ScTRP1* disrupted copy (Figure 11). Specifically, the P1

mutant lysogens harboring the Transfer plasmid and pac ABC complementing plasmid were growth at 32C and diluted 1:100 into fresh medium every 16 hours. DAN was extracted on day 1, 2, 3, 4, and 5, digested with HindIII and probed with a ScTRP1 EcoR1-BamH1 fragment under high stringency conditions.

5 In order to prevent reconstruction of functional (pacABC pac enzyme by recombination events between the Transfer plasmid and the modified P1 phage genome, silent mutations were introduced into the complementing plasmid as shown in Figure 12. Silent mutations in the complementing plasmid *pac* site lead to a defective *pac* site even if recombination occurred, and ensured that a defective pac site was be introduced into the P1
10 *pac* knockout (Figure 12). The 162 bp pac site is sufficient to promote pac cleavage and P1 packaging. The positions of the hexanucleotide elements with the HEX4 and HEX3 domains are shown by open boxes. the IHF binding site, consensus sequence 5'-AATCAANNANTTA, is indicated. Regulation of pac cleavage involves adenine methylation at 5' - GATC sites. Silent mutations introduced into the pac site are indicated
15 by lower case letters.

IN VIVO DELIVERY OF THERAPEUTIC AGENTS BY P1 VIRIONS

All five animal models listed in Table 6 are exemplified herein. LD50's have
20 been established in the peritonitis models for *E. coli* and *P. aeruginosa*, and the doses required are high (10^7 - 10^8 bacterial cells/animal). Further, a cystic fibrosis model of pseudomonas infection in mice is used to demonstrate efficacy of the toxic agents and methods of the invention for treatment of opportunistic lung infection characteristic of this disease.

25

Table 6. Animal Models for Prokaryotic Gene Therapy	
Animal Model	LD50
<i>E. coli</i> peritonitis	3×10^8 cfu
30 Pseudomonas peritonitis	1×10^7 cfu
Pseudomonas-burn in mice	<10 cfu
Pseudomonas-burn in rats	1×10^8 cfu
Pseudomonas in neutopenic mice	2×10^3 cfu

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PERITONITIS MODELS

A Transfer plasmid of the invention has been delivered with the P1 delivery system to *E. coli* and *Pseudomonas* *in vivo* in the mouse peritonitis model. Transfer was confirmed by re-isolation of the plasmid from bacteria recovered from the intraperitoneal space, and by restriction analysis of the recovered plasmid. Results demonstrate that the delivery vehicles of the invention are capable of delivering the toxic agents of the invention to a bacterial target without toxicity to the infected subject.

The immune response to the phage and phage clearance kinetics *in vivo* has also been examined. Results indicate that single injections of 2×10^9 lysogen forming units (lfu) of P1 phage per mouse resulted in the production of anti-phage antibodies in 8-14 days. Two groups of 4 mice were injected intraperitoneally (IP) with 2×10^9 lfu of long circulating P1 phage. Peripheral blood was sampled by tail clip at 1, 4, 8 and 24 hours post injection and titered with *E. coli* C600 target cells. The previously phage-challenged group had been injected IP with an equivalent dose of the same phage preparation 18 days prior to this experiment. The pre-immune group had no prior treatments. This resulted in rapid clearing of the phage *in vivo* (Figure 13). However, for human therapeutic considerations, many infected subjects (especially for pseudomonas) will be immunocompromised and incapable of generating strong immune responses. Accordingly, the Therapeutics and compositions of the invention may be particularly beneficial for such human subjects.

In addition, a long-circulating variant of P1 was selected by passage through mice that results in greater than 200 times more phage remaining in circulation at 24 and 30 hours after injection (Figure 14). Groups of 6 mice were injected IP with either 5×10^8 lfu P1 phage or 5×10^8 lfu long-circulating P1 phage. Peripheral blood was sampled by tail clip at 1, 6, 24, and 30 hours post injection and titered with *E. coli* C600 target cells. The number of viable phage remaining per ml of blood at each time-point is indicated in Figure 14. The fold improvement in persistence in the circulation is given in the last column of the table (lfu long-circulating P1 phage/lfu original P1 phage). Accordingly, use of the long-circulating P1 is within the scope of the invention. Such variant may be particularly preferable when increased concentrations of phage are desired in the circulation of an infected subject. For example, it may be desired in the case that the subject has a pathogen or bacterial infection in the blood.

EMBRYONATED HEN EGG MODEL

In order to demonstrate efficacy of the toxic agent delivered by the P1 delivery vehicle, an embryonated hen egg model of infection has been modified from published protocols. Superficially, the hen egg model of Hartl, A., et al., (1997, 5 "Pseudomonas aeruginosa infection in embryonated hen's eggs" *Arzneim.-Forsch.* 47(II): 1061-1064), was modified in the manner in which the eggs were incubated and the shells opened and administration was performed. Briefly, eggs were incubated in the vertical position, wide pole up, with automatic turning in a 90 degree arc every 4 h. Shells were opened on the wide pole end, by reinforcing the shell with adhesive tape and cutting a round 10 hole with a scissors through the tape and shell (opening diameter approx. 1cm). The underlying shell membrane was moistened with sterile water, then partially removed by tearing off a 1 cm² portion with a sterile forceps, which exposes the transparent chorioallantoic membrane (CAM). The shell was sealed against moisture loss with more adhesive tape and incubation continued for 18-24h. Viability was assessed at that time by 15 candling (observing the embryo by holding the egg in front of a bright light source). Observation of spontaneous movement was evidence of viability. Viable eggs were inoculated by pipeting bacterial suspensions onto the CAM. Therapeutic agents were pipeted onto the CAM or injected through the shell at other locations by syringe. Openings in the shell were resealed with tape, incubation continued, and viability was scored at 20 intervals by candling as above. Bacteria and phage were introduced into the egg through an opening made in the shell, which was then sealed and gestation continued.

An embryonated hen egg model was established, as above, which harbors a variety of advantages as an *in vivo* system. Specifically, the egg model required very low LD50 (<10 cfu/egg for *P. aeruginosa* and >50 cfu/egg for virulent strains of *E. coli*), the egg 25 model is also rapid, self contained and provides for an immature immune system. Human clinical isolates of *E. coli* and *P. aeruginosa* (PA01) consistently produce lethal infections in this model at very low doses of bacteria (100-1000 cells) allowing demonstration of the therapeutic agents. These tests show efficacy for the toxic agent such as *doc in vivo*.

In order to demonstrate the ability of the delivery vehicles of the invention to 30 deliver a Transfer plasmid *in vivo*, a Transfer plasmid carrying a kanamycin resistance gene was delivered to *E. coli* and *P. aeruginosa in vivo*, in mice and in embryonated hen eggs. Results indicated that delivery of toxic agent by the P1 system is successful *in vivo*.

***P. aeruginosa* Hen Egg Model**

In vivo plasmid transfer in chicken embryos: The Transfer plasmid pBHR was delivered to bacterial cells by P1 phage *in vivo* in using embryonated hen eggs, Specifically, groups of six embryonated hen eggs were inoculated via the chorioallantoic membrane on the tenth day of gestation with the bacteria and phage indicated. P1 lysogen which harbors *pDoc*, a transfer plasmid which encodes the *doc* gene. This phage preparation was a mixture of particles containing either p1 DNA or *pDoc*. Phage lysates were approximately a ratio of 99:1 P1 containing phage particles to *pDoc* containing particles.

Results demonstrated increased survival of eggs when P1 or P1-*pDoc* lysates are added immediately after inoculation with human clinical *P. aeruginosa* PA01 (Figure 15).

***E. coli* Hen Egg Model:**

A human clinical *E. coli* isolate which is refractory to transduction with P1 DNA has been found to produce a lethal infection in embryonated hen eggs. This isolate was designated EC-4, and is important for two reasons. First, since this strain cannot form a stable P1 lysogen, killing of EC-4 cells by *doc*-carrying phage preparations demonstrated the lethal activity of the toxic agent *doc*.

Specifically, P1-*pDoc* lysates killed EC-4 *E. coli* *in vitro* more efficiently than P1-pBHR phage alone (see Figure 16). Specifically, EC-4 cells (500 cfu) were treated with phage containing toxic agent *doc* (P1-*pDoc*) or control transfer plasmid pBHR (P1-pBHR) at the multiplicities of infection (MOI) shown in Figure 16, plated on non-selected media and counted as a percent of live cells treated with buffer alone. Results indicate that the toxic agent *doc* was able to render *E. coli* EC-4 less fit and increase killing of the pathogenic bacteria. Additionally, *E. coli* killing was confirmed *in vitro*: at a P1 MOI of 500-700, *doc* was able to kill the *E. coli* at a MOI 5-7, i.e. 1% of the total P1 particles (Figure 16).

Second, existence of this strain in a random sample of clinical isolates from local hospital demonstrated that there were pathogenic bacterial strains in the human population which were resistant to lytic phage therapy but susceptible to Toxic agent phage delivery system. Specifically, as shown in Table 7, below three clinical *E. coli* isolates were compared for their ability to be transduced with P1 DNA (as indicated by acquisition of chloramphenicol resistance) and transduced with transfer plasmid DNA (as indicated by acquisition of kanamycin resistance) relative to laboratory *E. coli* strain C600. All three clinical isolates were transduced with the Transfer plasmid, but only two became lysogenic

with P1. These results indicate that a phage resistant mechanism was preventing transduction of P1 viral DNA was unable to prevent delivery of the transfer plasmid to EC-4 cells.

5

Table 7, Clinical Isolates - susceptibility to P1 Transduction.

<i>E. coli</i>	Transduction with P1 DNA	Transduction with Transfer Plasmid DNA	
C600 (lab strain)	1.9×10^7 lfu	7.7×10^5 lfu	C600 in transduced with P1 DNA and the transfer plasmid
EC-1 (Human)	2.0×10^7 lfu	8.5×10^6 lfu	EC-1 in transduced with P1 DNA and the transfer plasmid
EC-2 (Human)	9×10^6 lfu	7.5×10^6 lfu	EC-2 in transduced with P1 DNA and the transfer plasmid
EC-4 (Human)	0	2.8×10^6 lfu	EC-4 in transduced with P1 DNA and the transfer plasmid

Further, an infection with 2×10^3 EC-4 cells was cured in eggs by a P1-*pDoc* lysate treatment given immediately after inoculation with the bacteria at a P1 MOI of 700-800 (*doc* containing virions were 1% of total phage particles) (Figure 17). Specifically, groups of seven embryonated hen eggs were inoculated via the chorioallantoic membrane on the tenth day of gestation with the bacteria and phage indicated. P1-*pDoc* phage was produced from a P1 lysogen which harbors *pDoc*, a transfer plasmid encodes the *doc* gene or control transfer plasmid pBHR. This phage preparation was a mixture of virions containing either P1 DNA or *pDoc*. Phage lysates were approximately a ratio of 99:1 P1 containing phage particles to *pDoc* containing particles. These results indicate that a pathogenic infection may be eradicated by a therapeutic of the invention, such as *doc* via a P1 delivery vehicle.

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MAMMALIAN ANIMAL MODELS

Three mouse and rat models are used to demonstrate the efficacy of the Toxic agents of the invention. Each models uses an immunocompromised animal, which is then followed by a bacterial challenge. The models differ in the route of bacterial challenge and
5 the means of producing the immune impairment.

In two models, immune impairment is produced in a burn model. Specifically, a burn of 10-20% total body surface area in humans or other animals results in a period of immune impairment, involving nearly all branches of the immune system, which lasts from 10-14 days. Two burn models, well documented in the literature
10 (see, e.g., J.P. Waymack, et al, 1988, "An evaluation of cyclophosphamide as an immunomodulator in multiple septic animal models" J. Burns and Clinical Rehabilitation 9(3):271-274; see also Stieritz, D. D. and Holder, I. A., 1975, "Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: Description of a burned mouse model" J. Infect. Dis. 131(6): 688-691) for experimental infections with
15 *Pseudomonas aeruginosa*, are used to demonstrate the effectiveness of a toxic agent therapeutic against the types of infections which occur with this type of wound.

The third model utilizes the biological modulator cyclophosphamide to produce an immunocompromised state (leukopenia), in which endogenous microflora of the intestinal tract can invade the body cavity and cause sepsis. This type of sepsis has been
20 documented in human patients with immunodeficiency (see, Furuya, et al ,1993, "Mortality rates amongst mice with endogenous septicemia caused by *Pseudomonas aeruginosa* isolates from various sources." J. Medical Microbiology 39: 141-146; Woods, et al, 1997, "Correlation of *Pseudomonas aeruginosa* virulence factors from clinical and environmental isolates with pathogenicity in the neutropenic mouse" Can. J. Microbiol. 43: 541-551).

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Model 1: Adult mice, dorsal burn, wound surface bacterial challenge

The first model of use is that of Stieritz, D. D. and Holder, I. A. (1975, J. Infect. Dis. 131(6): 688-691); also see Neely, A.N. and Holder, I. A., 1996, "A murine model with aspects of clinical relevance for the study of antibiotic-induced endotoxin
30 release in septic hosts. J. Endotoxin Research 3: 229-235.). Young adult female mice, 22-25g, ICR strain (or possibly Balb/c, CD1, C3HEB/FeJ, C3H/HeJ, C57BL/6, DBA/2, A/J, CBA, C3H/HeN) are anesthetized with pentobarbitol and shaved of dorsal hair. A heat resistant plastic card with a 1 x 1.5 inch opening is placed on the shaved back, 0.5 ml ethanol pipetted onto the exposed skin and ignited for a 10 second burn. The flame is
35 extinguished, and the mouse given 1-2 ml saline via intraperitoneal (IP) injection as fluid replacement. This procedure produces a non-lethal, partial thickness burn covering 12-15%

of the body surface area of a 22-25g mouse (Neely and Holder, 1996, supra). One hour after the burn, and after mice have received analgesia (buprenorphine 2 mg/kg, IM), a small inocula of bacteria (100 cfu *P. aeruginosa*) in 0.1 ml saline is injected subcutaneously into the wound. Toxic agent treatment agent or placebo is administered either simultaneously to the same site (also 0.1 ml in saline) or by IP injection (up to 0.5 ml in saline) 1 hour after or shortly before challenge. Animals are observed for sepsis and medicated for pain (buprenorphine 2 mg/kg, IM) at intervals not exceeding 12h. Normal diet and water is provided ad libitum. Mortality is expected in untreated burned groups within approximately 48h. Blood samples (10-25 ul) may be taken at 12-24h intervals by tail bleed to monitor bacterial load. Blood and organs are collected at time of death or euthanasia, to monitor bacterial load and confirm death from *P. aeruginosa* sepsis or clearance of infection in treated animals.

Model 2: Adult rat, dorsal burn, IP or wound surface bacterial challenge

The second model is that of Waymack et al. (J.P. Waymack, G.D. Warden, J.W. Alexander, P.M. and S. Gonce, 1988, "An evaluation of cyclophosphamide as an immunomodulator in multiple septic animal models". *J. Burns and Clinical Rehabilitation* 9(3):271-274.). Young male Lewis rats (100-125g) are anesthetized by IP pentobarbital injection (~ 40 mg/kg) and shaved of dorsal hair. The animal is pressed against a heat resistant template that exposes the shaved area (20% of the total body surface area). This template is immersed in a 95°C waterbath for 10 seconds. After removal from the waterbath, the animals receive 5-10 ml Ringer's Lactate solution by IP injection for fluid maintenance therapy (approximately one half blood volume is recommended) and buprenorphine for analgesia (0.1-0.5 mg/kg, every 12h). This injury is reported to be a full-thickness burn resulting in zero mortality in the absence of further injury. A 50% lethal dose bacterial challenge (1×10^8 cfu *P. aeruginosa* in 0.5 ml saline) is introduced by IP injection on day 4 post-burn or by painting the bacterial suspension on the wound on day 1 post-burn. The IP infection route is reported to produce sepsis within 24h (i.e. day 5 post-burn) with all deaths occurring by day 12 post-burn. Therefore, an IP injection demonstration may be terminated on day 12 post-burn. Painting of *Pseudomonas* on the burn is reported to result in sepsis 7-8 days after inoculation (day 8-9 post-burn), and survival rates are stable by day 20. Euthanasia of animals subjected to this regimen will be on day 21 post-burn. Normal diet and water will be provided ad libitum. Some animals are treatment with the therapeutic of the invention (P1 phage comprising a Transfer plasmid encoding a toxic agent) which is administered topically to the burn region or by IP injection. Blood samples (50 100 ul) may be taken by retro-orbital bleed of pentobarbital anesthetized

rats at intervals of 12-24h to monitor bacterial load. Blood and organs are collected at time of death or euthanasia, also to monitor bacterial load and confirm death from *P. aeruginosa* sepsis.

5 **Model 3: Adult mouse, antibiotic and cyclophosphamide injections, oral bacterial challenge**

This is the model of endogenous septicemia of Furuya et al.(Furuya, N., Hirakata, Y., Tomono, K., Matsumoto, T., Tateda, K., Kaku, M., and Yamaguchi, K. ,1993, "Mortality rates amongst mice with endogenous septicemia caused by *Pseudomonas aeruginosa* isolates from various sources." J. Medical Microbiology 39: 141-146). Mice weighing 20-25g are housed in a sterile environment (e.g., in an isolator) and given sterile diet and water. IP injections of sodium ampicillin (200mg/kg) are given on days 1 and 2 to disturb normal intestinal flora and aid colonization by *P. aeruginosa*. Cyclophosphamide is injected IP (250 mg/kg) on days 6 and 9. This dose induces leukopenia without lethality in the absence of infection. The bacteria are administered to the mice in their drinking water on days 2-4. Treatment with therapeutic of the invention (P1 phage comprising a Transfer plasmid encoding a toxic agent) is started on day 9, and is administered by IP injection. Fecal pellets are be collected before bacterial challenge and at intervals throughout the infection to monitor for the absence and presence of *P. aeruginosa*. The onset of sepsis is expected 24-48 h after the second dose of cyclophosphamide (day 11), and approximately 80% mortality is expected by day 14. Signs of distress in the animals are treated with buprenorphine (2 mg/kg, twice daily or as needed). Blood samples obtained by tail bleed may also be taken at 12-24 h intervals after day 4. Alternatively, the ampicillin injections can be avoided by introducing the bacteria by IP injection the day after the final cyclophosphamide injection (Woods, D.E., Lam, J.S., Paranchych, D.P., Speert, D.P., Campbell, M., and Godfrey, A.J. ,1997, "Correlation of *Pseudomonas aeruginosa* virulence factors from clinical and environmental isolates with pathogenicity in the neutropenic mouse. Can. J. Microbiol. 43:541-551).

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35

Table 8: Therapeutic formulations in the following format used in the mouse models:

Group	Challenge	Phagemid treatment	Approximate # of survivors
1	Burn or cyclophosphamide (cyc)	1000 x moi agent 1	6/6
2	Burn or cyc	1000 x moi agent 2	6/6
3 - 8	Burn or cyc	1000 x moi agent (n)	6/6
9	Burn or cyc +LD ₁₀₀ pseudomonas	None	0/6
10	Burn or cyc +LD ₁₀₀ pseudomonas	1000 x moi agent 1	0/6 6/6
11	Burn or cyc +LD ₁₀₀ pseudomonas	1000 x moi agent 2	0/6 6/6
12 - 17	Burn or cyc +LD ₁₀₀ pseudomonas	1000 x moi agent 3	0/6 6/6

Total: up to 112 mice/ model X 2 models= 224 mice

Table 9: A dose response demonstration is performed as follows:

Group	Challenge	Phagemid treatment	Approximate # of survivors
1	Burn or cyclophosphamide (cyc)	1000 x moi	6/6
2	Burn or cyc +LD ₁₀₀ pseudomonas	None	0/6
3	Burn or cyc +LD ₁₀₀ pseudomonas	1000 x moi	0/6 6/6
4	Burn or cyc +LD ₁₀₀ pseudomonas	100 x moi	0/6 6/6
5	Burn or cyc +LD ₁₀₀ pseudomonas	10 x moi	0/6 6/6
6	Burn or cyc +LD ₁₀₀ pseudomonas	1 x moi	0/6 6/6

Total: up to 36 mice/ model/agent x 8 agents x 2 models = 576 mice

Therapeutic

Table 10: Therapeutic formulations in the following format are used in the rat

models:

Group	Challenge	Phagemid treatment	Approximate # of survivors
1	Burn	1000 x moi	6/6
2	Burn +LD ₁₀₀ pseudomonas	None	0/6
3	Burn +LD ₁₀₀ pseudomonas	1000 x moi	0/6 6/6
4	Burn +LD ₁₀₀ pseudomonas	100 x moi	0/6 6/6
5	Burn +LD ₁₀₀ pseudomonas	10 x moi	0/6 6/6
6	Burn +LD ₁₀₀ pseudomonas	1 x moi	0/6 6/6

Total: up to 36 rats /agent x up to 8 agents = 278 rats

Results of animal demonstrations indicate that the phage therapeutics comprising a toxic agent of the invention, is suited to treat bacterial infections of a subject. Other animal models known in the art are within the scope of the invention, including but not

limited to models using calves, pigs, lambs, guinea pig, rabbits, etc. In a preferred aspect of the invention, the subject in need of a therapeutic of the invention is a mammal with a burn injury.

5 **TREATMENT OF OPPORTUNISTIC INFECTIONS IN** **A MURINE MODEL OF CYSTIC FIBROSIS**

4 The toxic agents of the invention are useful for the treatment of pathogenic
 infection such as infections associated with ~~cystic fibrosis~~ ^{cystic fibrosis}. As demonstration of the
 unility of the invention, a mouse model of pseudomonas respiratory infection is used
10 which mimics the type of infection seen in human Cystic Fibrosis (CF) patients. This
 model uses adult (6-8 week old) mice which carry the DF508 mutation in the cftr gene
 (C57BL/6 DF508 mice) and their wild type counterparts (C57BL/6 mice), or BALB/c
 adult mice without cftr mutations. The DF508 mutation is one of the most common
 mutations found in human CF patients, and the C57BL/6 DF508 mice have many
15 symptoms similar to humans with this disease. After weaning, DF508 cftr homozygous
 mutants must be maintained on a liquid diet of Peptamin (Clintec Nutrition Co., Deerfield,
 MI) and water containing golytely (Braintree Laboratories, Braintree, MA) in order to
 prevent fatal bowel obstructions which are common in these mice due to their cftr
 mutation (see Zaidi, T. S., et al, 1999 "Cystic fibrosis transmembrane conductance
20 regulator-mediated corneal epithelial cell ingestion of Pseudomonas aeruginosa is a key
 component in the pathogenesis of experimental murine keratitis" Infection and Immunity
 67(3): 1481-1492). BALB/c mice can also be used if C57BL/6 DF508 mice are not
 available.

 The experimental procedure is as follows (see e.g., Pier, G.B. et al.,1996,
25 "Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections"
 Science 271: 64-67). Adult mice are anesthetized by intraperitoneal injection of a freshly
 prepared mixture of ketamine hydrochloride (65 mg/kg) and xylazine (13 mg/kg). Then
 with mice are held in an upright position, and 10 ul of a bacterial suspension is placed in
 each nostril (20 ul total). Mice are allowed to regain conciousness and then either
30 observed for survival for up to 72 hours, or, sacrificed by CO₂ overdose at various time
 periods up to 24 hours after infection for determination of bacterial loads in various
 tissues, especially the lungs. Anesthesia is a necessary part of the infection procedure.
 Unanesthetized mice fail to aspirate the inoculum efficiently and do not become infected.
 Therapeutic phage comprising one or more toxic agents of the invention are administered,
35 for example, intranasally, intravenously, or intraperitoneally. Mice administered the
 Therapeutic of the invention survive longer than the untreated control mice. Accordingly,

the toxic agents of the invention may be delivered to a subject harboring a pathogenic (e.g., bacterial) infection for the purpose of ameliorating or eradicating the infection.

8. EXAMPLE: CONSTRUCTION & CHARACTERIZATION OF *SO^F* SENSE RNA AS A TOXIC AGENT

In order to demonstrate that a toxic agent may be delivered and expressed using a ribozyme cassette, the inventors have engineered a toxic agent directed against an essential molecule called *Sof*, and delivered the toxic agent in a ribozyme cassette to bacterial cells to cause the death of the bacterial cells.

As described herein above, a toxic agent may be a molecule which is designed to target an essential molecule of a pathogen or selected cell. An example of an essential antisense molecule for bacteria is *Sof*. *Sof* is an antisense antidote for a chromosomally encoded toxin called *gef*. *Sof* normally acts to regulate the levels of *gef* in the pathogen, and thus allows the cell to survive in the presence of *gef*. The inventors of the present invention have designed sense molecules which are complementary to *Sof*. The sense molecules against *Sof* acted to inhibit the ability of *Sof* to regulate *gef*, and thus caused toxicity in the pathogen by allowing the endogenous *gef* levels to become toxic to the bacteria.

Specifically, *Sof* sense was constructed into a triple ribozyme cassette (with 5' and 3' *cis*-acting ribozymes). The ribozyme cassette containing the *Sof*^{sense} toxic agent was linked to the LEASHI promoter. The nucleic acids encoding the ribozyme cassette were then used to transform *E. coli*. Bacterial cells were plated onto LB Amp + IPTG. Plates were incubated overnight at 37°C. Plates were then scored for the presence of transformants, size of colonies, growth rate, and morphological differences.

Results of these studies indicated that expression of the *Sof* sense molecules from the ribozyme cassette lead to toxic effects in the targeted bacteria.

9. EXAMPLE: RIBOZYMES AND RIBOZYME CASSETTES

The ribozyme cassettes which are particularly useful in the methods of the invention include but are not limited to the following:

pClip (the genetic element described in Figure 19) is a modification of pBluescript, wherein the cassette shown is dropped into the *Not I* site in pBluescript. The toxic agent or trans-acting ribozyme is constructed into the *Bgl II* site (TGCTCT). Liberation of internal ribozymes or toxic agents from pClip results in a distribution of the toxic agent or ribozyme(s) to approximately 20% nuclear and 80% cytoplasmic, when delivered to a eukaryotic cell. pClip is also used to target prokaryotic cells.

A second ribozyme cassette/vector that is useful in connection with the methods of the invention is pChop. pChop is modified from pClip to convey a more efficient and effective liberation of the internal trans-acting ribozymes or toxic agents. The pChop ribozyme cassette is diagramed in Figure 20. Liberation of internal catalytic core ribozymes from pChop increases localization to the nucleus when delivered to a eukaryotic cell.

A third ribozyme cassette that was useful in connection with the methods of the invention is pSnip. The pSnip multi-ribozyme is constructed by engineering the pClip cassette 5' to pChop. In addition, the pSnip multi-ribozyme contains catalytic core sequences with two trans-acting ribozymes or toxic agents in each cassette. Each pair of trans-acting ribozymes or toxic agents is linked by a short spacer and stabilized by a hairpin loop located 3' to the pair. Figure 21 diagrams the schematic of the pSnip cassette.

A trans-acting ribozyme, or antisense toxic agent is synthesized as reverse complementary overlapping oligodeoxynucleotides, which are designed in such a way that when annealed they form single stranded ends identical to those produced by digestion with the restriction endonuclease contained with the region between the two cis-acting ribozymes. In this particular example the restriction endonuclease recognition site is that recognized by *Bgl* II. Essentially any RNA can be targeted: specificity is conferred by selecting sequences for the ribozyme that are reverse and complementary to sequences flanking the chosen cleavage site in the targeted RNA molecule. The toxic agent(s) or trans-acting ribozymes are then cloned into the cloning region(polylinker) within the double ribozyme cassette to produce the targeted toxic agent or ribozyme. Trans-acting ribozymes targeted to prokaryotic sequences have been constructed including, but not limited to, *Escherichia coli*: *secA* (EcosecA, AE000119 U00096) , gene X (EcosecA, AE000119 U00096) *ftsZ* (AE000119;U00096) , *dnaG* (AE000388 U00096) , *rpoA*(AE000407 U00096) and tRNA-asf (X14007) , *Streptomyces lividans secA* (Z50195) , *Enterococcus faecalis*, *ftsZ* (U94707) *Pseudomonas putida*, *dnaG* (U85774), *Streptomyces coelicolor rpoA* (X92107), *Staphylococcus warneri* tRNA-Asp (X66089 S42075), *Staphylococcus* RNA III.

The utility of the design using eukaryotic sequences has also been evaluated; a) repetitive B2 transcripts (B2); b) RNA polymerase I (polI); c) Hepatitis B virus (HBV); d) Sonic Hedgehog (SH); e) Human Papillomavirus E6/E7 protein (HPV); f) RNA polymerase II (polII); g) Insulin-like Growth Factor 1 (IGF1); h) retinoblastoma protein (RB); i) and j) Multicatalytic Proteinase alpha-subunits C3 and C9 (C3 and C9, respectively); k) telomerase (tel); l) Transforming growth factor beta (TGF β); m) catalase (CAT); n) Peroxisome proliferation associated receptor (Ppar α); and o) Cytochrome P₄₅₀

1E1 (p4501E1); KiSS-1, NudC, Androgen Receptor, and SF-1 transcription factor. Target RNAs (with locus names and accession numbers) as well as the selected target sites are presented (Table 11).

5 **TABLE 11. Summary of Targeted RNAs and Target Sites**

	Target RNA	EMBL Locus	Accession	Target Site	Functional Testing	
					<i>in vitro</i>	<i>in vivo</i>
10	pol II	HSRNAP14K	Z27113	GTC ₈₃	ND	ND
	HBV	XXHEPAV	X02496	GTC ₄₃₈	IP	+
	HBV		X04615	GTT ₁₉₄₄	+	+
	HBV		X02496	GTT ₁₉₄₆	+	+
	HCV		M62321.1	GTC ₃₂₅	+	+
15	RB	MUSP105RB	M26391	GTC ₂₆₄	+	+
	IGF1	HUMIGF1B	M37484	GTC ₁₈₅	ND	ND
	SH	MMEVX1	X54239	GTC ₅₅₈	IP	IP
	Pol I	MUSRPA40	D31966	GTC ₄₅₈	+	+
	HPV	PPH16	K02718	GTT ₁₀₈	IP	+
20	C3	RATC3AA	J02897	GTT ₂₂	+	+
	C9	RNPTSC9	X533304	GTC ₁₀₁	+	+
	B2	B2-Consensus	##	GTT ₂₄	+	+
	Tel	MMU33831	U33831	CTA ₆₃	ND	ND

25 **Table 12: Sites Identified on HPV E6/E7 Target RNAs by Library Selection**

	HPV strains	GenBank Accession Number	Nucleotide Triplet and Position
30	HPV11	M14119	CTC121
	HPV16	K02718	TTC110
	HPV18	X05015	ATC123
	HPV11	M14119	ATA443
	HPV16	K02718	GTC437
	HPV18	X05015	ATC444
	HPV11	M14119	GTC507
	HPV16	K02718	GTC506
35	HPV18	X05015	ATA507

Table 13: Sites on HBV RNA Identified by Library Selection
Nucleotide Triplet and Site

Sites on HBV RNA	GenBank Accession Number
GTC1473	X02496
CTC1534	X02496
CTC1532	X04615, + *
ATC1842	X02496
ATC1840	X04615, + *
GTT1946	X02496
GTT1944	X04615, + *
CTC1950	X02496
CTC1948	X04615, + #
TTC1948	X75663, X75658

* Includes but is not limited to X75664, X75657, X75665, X75663 and X75658.

Includes but is not limited to X75664, X75657, X75656, and X75665.

10. EXAMPLE: DELIVERY AND IN VIVO TESTING

Biologic Delivery

The toxic agents and/or ribozymes of the present invention may be delivered by a wide variety of viral vectors and bacteriophage as described herein, and expemplified herein above.

In one embodiment of the invention, a toxic agent is encoded in a Transfer plasmid, and is used in connection with a P1 bacteriophage delivery system. Such Transfer plasmid preferably contains 1) an origin or replication 2) selectable marker 3) P1 PAC site and PAC ABC genes 4) P1 lytic replicon 5) nucleic acids encoding one or more toxic agents of the invention. In a preferred embodiment of the invention, the bacteriophage P1 prophage (P1 plasmid) is engineered such that viral DNA can not be packaged into virions, such as, for example, by deletion of the PAC site from the P1 plasmid.

In another embodiment, the toxic agents and/or ribozymes may be delivered via a plasmid encoding the toxic agents and/or ribozymes, a plasmid origin of replication, a selectable marker for plasmid maintenance, the minimal lambda origin of replication, and cos sites, which are required for packaging of DNA into lambda virions. This plasmid is maintained in a lambda lysogen that is defective in integration/excision and recombination functions. The defective lysogen provides all of the replication factors needed to activate the lambda origin of replication on the plasmid and all of the structural components needed to form mature virions; however, the lysogen is not able to replicate and package its own DNA into the virions. The lysogen also carries the cI^{857} temperature-

5 sensitive repressor mutation. Induction of the lysogen by temperature shift to 42°C or by other means, such as exposure to 5J/m2 of ultraviolet radiation will mobilize the plasmid and result in its replication and packaging into lambda virions. The virions can then be harvested, purified free of *E. coli* proteins and be used to deliver the toxic agents and/or ribozyme gene(s) to *E. coli*. Similar methods are performed for *Pseudomonas aeruginosa* in order to deliver a toxic agent and/or ribozyme to *P. aeruginosa*.

Abiologic Delivery

10 Abiologic delivery of the toxic agent and/or ribozymes is accomplished with constructs that have been engineered to be expressed within the targeted tissue or pathogen. Briefly, the genetic element containing the promoter and the toxic agent and/or ribozyme(s) are complexed with cationic liposomes (Lipofectamine--Gibco BRL) in a 1:10 ratio and are introduced into test animals by either single or multiple injection of 0.2 ml total volume nucleic acid-liposome mixture.

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Prophylactic Administration and Prevention of Acute Bacterial Infection

Following the demonstration that toxic agents and/or ribozymes of the present invention have an *in vitro* biological activity (either directly on bacterial cultures or in an infectious tissue culture cell assay system), the effectiveness of the toxic agents and/or ribozymes, is shown in *in vivo* model systems, *e.g.*, as described above. To demonstrate the efficacy of toxic agents and/or ribozymes of the invention *in vivo*, experimental animal model systems are utilized, such as those described herein. For an initial demonstration of the efficacy of the toxic agents and/or ribozymes *in vivo*, mice are infected with a microbial pathogen which has previously been shown to be sensitive to the toxic agents and/or ribozymes construct(s) and the effect of toxic agents and/or ribozymes administered *in vivo* is determined. In the first series of *in vivo* trials, one determines the effectiveness of toxic agents and/or ribozymes at preventing an acute infection in a murine model system when the toxic agents and/or ribozymes is added directly to the microbe prior to administration *in vivo*.

30 The next series of trials demonstrates that the administration of toxic agents and/or ribozymes after infection is effective at preventing an acute bacterial infections. In addition to the clinical status of infected mice, tissues obtained at necropsy are examined histologically and the presence of replicating microorganism in tissue samples is determined by standard methodology. Animals can be infected by various routes (systemic and/or mucosal) and the toxic agents and/or ribozymes are delivered over time after infection by systemic, mucosal, or topical routes. Both abiologic as well as biological

delivery of the toxic agents and/or ribozymes is used. The demonstration of a positive effect of the toxic agents and/or ribozymes in controlled experimental model system provides compelling evidence for the efficacy of the preparation and determines whether or not the preparation warrants evaluation under conditions of standard clinical trials.

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Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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